# Diagnostic Gene Panel Testing in (Non)-Syndromic Patients with Cleft Lip, Alveolus and/or Palate in the Netherlands

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**Abstract**

***Objectives:*** Clefts of the lip, alveolus and/or palate (CLA/P) are the most common craniofacial congenital malformations in humans. These oral clefts can be divided into non-syndromic (isolated) and syndromic forms. Many cleft-related syndromes are clinically variable and genetically heterogeneous, making it challenging to distinguish syndromic from non-syndromic cases. Recognition of syndromic/genetic causes is important for personalized tailored care, identification of (unrecognized) comorbidities, and accurate genetic counseling. Therefore, next generation sequencing (NGS)-based targeted gene panel testing is increasingly implemented in diagnostics of CLA/P patients. In this retrospective study, we assess the yield of NGS gene panel testing in a cohort of CLA/P cases. ***Methods:*** Whole exome sequencing (WES) followed by variant detection and

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interpretation in an a priori selected set of genes associated with CLA/P phenotypes was performed in 212 unrelated CLA/P patients after genetic counseling between 2015 and 2020. Medical records including family history and results of additional genetic tests were evaluated. ***Results:*** In 24 CLA/P cases (11.3%), a pathogenic genetic variant was identified. Twenty out of these 24 had a genetic syndrome requiring specific monitoring and follow-up. Six of these 24 cases (25%) were presumed to be isolated CLA/P cases prior to testing, corresponding to 2.8% of the total cohort. In eight CLA/P cases (3.8%) without a diagnosis after NGS-based gene panel testing, a molecular diagnosis was established by additional genetic analyses (e.g., SNP array, single gene testing, trio WES). ***Conclusion:*** This study illustrates NGS-based gene panel testing is a powerful diagnostic tool in the diagnostic workup of CLA/P patients. Also, in apparently isolated cases and non-familial cases, a genetic diagnosis can be identified. Early diagnosis facilitates personalized care for patients and accurate genetic counseling of their families.

## Introduction

Clefts of the lip, alveolus and/or palate (CLA/P) are a heterogeneous group of birth defects. Their common denominator is malfunctioning of fusion and/or differentiation processes during embryological development. These processes are driven by similar but not necessarily identical genetic and molecular pathways during the 6–12th week after conception [Luijsterburg et al., 2014; Losa et al., 2018; Welsh et al., 2018]. Therefore, full bilateral cleft lip and alveolus may exist in combination with a completely intact secondary palate and vice versa [Leslie and Marazita, 2013; Luijsterburg et al., 2014; Losa et al., 2018; Bishop et al., 2020].

Approximately 70% of cleft lip with or without cleft palate (CL/P) cases are classified as non-syndromic isolated cases. Of these, 80% are sporadic (single patient) cases and 20% are multiplex (familial) cases [Mossey and Modell, 2012]. For cleft palate only cases, it is estimated that half of these have a syndromic etiology [Leslie and Marazita, 2013]. Non-syndromic CL/P cases are considered to have a multifactorial inheritance pattern [Garland et al., 2020; Reynolds et al., 2020]. In familial non-syndromic CLA/P cases, however, a major genetic contributor might be the underlying cause [Basha et al., 2018].

Syndromic CLA/P cases are more likely to be caused by a single pathogenic gene variant. CLA/P is a clinical feature reported in over 400 Mendelian disorders, many chromosomal disorders, embryopathies due to teratogens, and in conditions with multiple congenital defects of unknown etiology [Leslie and Marazita 2013; Beaty et al., 2016; Conte et al., 2016; Garland et al., 2020; Reynolds et al., 2020; OMIM-database, www.OMIM.org]. For over 140 Mendelian disorders, the (genetic) etiology is currently not known (e.g., Schilbach-Rott syndrome [SRS, OMIM%164220] [www.OMIM.org]).

CLA/P can affect multiple domains related to appearance, feeding, speech, hearing, and socialization skills. State of the art care for children with CLA/P therefore requires a multidisciplinary approach. This ensures optimal monitoring and follow-up parallel to the surgical interventions between 0 and 18 years of age and guides transition of care into adulthood if needed [Worley et al., 2018; Sandy et al., 2020]. Despite adequate treatment, CLA/P may have long-lasting impact on the patients' health and social lives [Christensen et al., 2004; Corrêa de Queiroz Herkrath et al., 2018]. The intensity of treatment and monitoring of comorbidities depends on the type of oral cleft and its etiology.

Early identification of cases with a syndromic and/or genetic diagnosis is, therefore, pivotal to secure tailored care and long-term management [Stock et al., 2019]. However, differentiating non-syndromic CLA/P from syndromic CLA/P cases on clinical features only can be challenging. This holds even stronger for CLA/P cases that are detected antenatal. Due to increasing sensitivity of prenatal ultrasound technology, CLA/Ps are more frequently detected nowadays [Johnson 2019].

Especially in prenatally detected CLA/P and in newborns with CLA/P, absence of recognizable additional clinical features can impede early syndrome diagnosis and subsequently delay necessary therapeutic interventions. Additional clinical or developmental anomalies associated with syndromic CLA/P may only appear at a later stage in life [van der Veen et al., 2006; Rozendaal et al., 2021; Setó-Salvia and Stanier, 2014]. Differentiation between non-syndromic and syndromic CLA/P is not only important for optimal care of the individual patient but also provides important information on recurrence risks for future pregnancies and informed decision-making [Dixon et al., 2011]. Genetic counseling and screening of patients with CLA/P is therefore becoming increasingly important and is more routinely advised, both pre- and postnatally, in the Netherlands [Mink van der Molen et al., 2021].

Marazita and colleagues offer a comprehensive overview of the evolution of human genetic studies of CLA/P [Marazita, 2012]. Next generation sequencing (NGS),

including whole exome sequencing (WES), proves to be highly efficient and cost-effective in analyzing many genes simultaneously [Tan et al., 2017; Bouman et al., 2018; Manickam et al., 2021]. However, analysis of the entire exome has some limitations in cases with an oral cleft phenotype. It often leads to the identification of many variants with unknown significance, requiring additional clinical evaluation and segregation analyses within the family. Only by including the parents in exome trio analyses, the number of identified familial non-pathogenic variants can be reduced. Such inheritance-based analyses, however, can potentially filter out pathogenic (autosomal dominant) variants in, for example, CL/P-associated genes with reduced penetrance or phenotypic variability inherited from an unidentified affected/healthy carrier parent. Possibly even more important, whole exome analysis might lead to overdiagnosis by uncovering unsolicited findings which predispose to a disease unrelated to the clinical question. Therefore, first tier analysis of a highly curated set of genes associated with CLA/P phenotypes is nowadays common in clinical genetic counseling of CLA/P in the Netherlands.

The primary aim of this retrospective study was to evaluate the diagnostic yield of WES followed by variant detection and interpretation in an a priori selected set of genes associated with CLA/P phenotypes in a cohort of patients with CLA/P whom were referred to a clinical geneticist. The second aim was to address the value of cleft gene panel testing in better differentiating non-syndromic from syndromic CLA/P cases and in identifying the specific underlying diagnosis in clinically syndromic cases.

## Method

### Study Design and Population

This study was initiated at the Department of Genetics, University Medical Centre Utrecht (UMCU), as a multi-center retrospective cohort study. The cohort comprises 212 unrelated index CLA/P cases in which NGS cleft gene panel testing was part of the clinical genetic workup.

Patients presented at the genetics department of one of the contributing university medical centers: University Medical Center Utrecht, Amsterdam University Medical Center, Leiden University Medical Center, University Medical Center Groningen, Erasmus MC University Medical Centre, and Maastricht University Medical Center. Genetic analyses were performed between January 2015 and December 2019.

All patients agreed on diagnostic NGS-based gene panel testing of cleft-related genes. Ethical approval for this retrospective study and a waiver of informed consent was obtained from the UMCU Ethical 320 Committee (local number: 19-061/c).

All patient information was anonymized and de-identified before entry into the database and before analysis. Data were stored on password-protected hospital computers.

### Cohort Description

A total of 212 cases (105 females, 107 males) were included, most of whom were recruited at the UMCU (129 cases, 60.8%). Patients with and without suspected syndromic orofacial clefts were included. The oral cleft was classified according Luijsterburg and colleagues [Luijsterburg et al., 2014]. The following three subgroups were defined: (1) CL/A, cleft lip with or without alveolus ($n = 25$); (2) CL/AP, cleft lip with or without alveolus and cleft palate ($n = 77$); (3) CP, cleft palate only ($n = 103$). In 7 cases, the exact cleft classification was not available.

The CL/AP group comprised 2 cases with features of Robin sequence ($n = 2/77$, 2.6%). The CP group comprised 27 cases with features of Robin Sequence ($n = 27/103$, 26.2%) and a subgroup with velopharyngeal insufficiency (HP:0000220)/congenital velopharyngeal incompetence ORPHA:2291 (VPI) ($n = 7/103$, 6.7%). The reason to include these VPI cases was that their VPI was presumed to be caused by a submucous cleft of the palate or hypoplastic palate [Glade and Deal, 2016].

### Data Availability, Patient and Public Involvement

Genetic testing was part of the diagnostic workup in the clinic, which included detailed physical examination by an experienced clinical geneticist. The DNA analyses was performed after extensive genetic counseling and based on shared decision-making. The data on gene panel testing, where we report on, are partly derived from WES-based data analysis, which is part of current clinical practice. While the data were obtained in clinical care, patients were not part of a research cohort or biobank and therefore did not give their consent to making their complete genetic data publicly available.

### Data Collection

For data collection, a case report form using HPO terms was composed in Castor EDC [Merlin et al., 2018; Köhler et al., 2021]. The medical history was assessed by the local clinical geneticist, who extracted the data from the medical records in their respective center. The following parameters were obtained: demographics (age – at the time of request for diagnostic gene panel testing, sex), medication during pregnancy, comorbidities, and morphological abnormalities [Köhler et al., 2021], focusing on the presence of major abnormalities, minor anomalies, and anomalies related to specific cleft-related syndromes (online suppl. Table. S1; for all online suppl. material, see www.karger.com/doi/10.1159/000530256). Previous studies showed that the presence of three or more minor anomalies in newborns makes the presence of a major malformation more likely [Merks et al., 2003; Leppig et al., 1987]. Therefore, the presence of three or more minor anomalies was scored in each patient (MJB). Major abnormalities (malformations; defect of embryogenesis or other abnormalities; deformation, disruption, dysplasia) and minor anomalies (with a prevalence ≤4% in general population) were defined according to the definition reported by Merks and colleagues [Merks et al., 2003, 2006].

Family data were obtained from the medical records. Finally, all results of the reported genetic tests were collected.

In case of doubt regarding interpretation of information provided by the clinical geneticists in the case report form, the reporting clinical geneticist was contacted and/or a meeting was held with a clinical geneticist (MJB), laboratory specialist (MM), and plastic surgeon (AMM) to reach consensus.

*Genetic Testing*
NGS-Based Gene Panel Testing
After enrichment of the exome with the Agilent SureSelect V6/CREV2 kit (Agilent Technologies, Santa Clara, CA, USA), WES was performed on an Illumina Nextseq or Novaseq 6000 sequencer at the section of Genome Diagnostics, Department of Genetics, University Medical Center Utrecht. The Illumina sequencing data were processed with incremental versions of our in-house developed pipeline, IAP v2. x.x [1], including GATK according to the best practices guidelines. Briefly, we mapped the read pairs with BWA-MEM marked duplicates and merged lanes using Sambamba and realigned indels using GATK Indel-Realigner. Next, GATK Haplotypecaller was used to call SNPs and indels to create VCF formatted files. For gene panel analyses, the aim is a minimal coverage of more than 15 unique reads for 99% of the bases in protein-coding exons and flanking splice-site consensus sequences of all genes included in the panel. This method detects more than 95% of the variants in the gene panel (Robert F Ernst, Mark van Roosmalen, Joep de Ligt, Sander Boymans, Roel Janssen, and Isaac J Nijman (2017, November 1). UMCUGenetics/IAP: v2.6.1. Zenodo; https://zenodo.org/record/3744169#.Y6Rmx7qZOUk).

The cleft gene panel (including 162–252 genes, depending on versioning) for this study was initially developed by the Genetics Department, UMCU, and was updated approximately once a year. The clinical synopsis provided by the OMIM database served as the basis for the panel. Adaptations were made after multidisciplinary consultation with clinical geneticists and molecular genetic laboratory specialists and literature searches for novel cleft genes in PubMed (http://pubmed.ncbi.nlm.nih.gov). Because of the diagnostic scope of this cleft gene panel, in subsequent versions of the gene panel, candidate genes and genes reported in single cases and/or without functional confirmation/substantiation were left out.

The latest update was drafted with aid of the Genomics England PanelApp version 1.38 (http://panelapp.genomicsengland.co.uk/panels/81). The different gene panel versions (OWS02v17.1/17.2/19.1/20.1) can be found in online suppl. Tables S2–S6.

*Variant Interpretation and Classification*
To systematically predict pathogenicity, the identified variants were classified according to the existing American College of Medical Genomics guidelines [Richards et al., 2015]. Variants are classified as benign (class 1), likely benign (class 2), variant of uncertain clinical significance (class 3), likely pathogenic (class 4), and pathogenic (class 5). Variant annotation, prioritization, and interpretation were performed with Agilent Allissa Interpret (https://www.agilent.com/cs/library/casestudies/public/5991-8533EN.pdf). Reclassification of variants was performed after careful re-evaluation of the putative diagnosis, molecular findings, and/or segregation analyses in relevant family members.

*Additional Genetic Testing*
It was reported when additional genetic testing (e.g., SNP array, other gene panels, trio WES) was performed as part of the diagnostic workup, and an underlying diagnosis was established.

*Statistical Analysis*
Standard descriptive statistics were used to summarize characteristics of the study population. Normality of data was tested with the Shapiro-Wilk test. Continuous data were presented as proportions, mean (±SD), or median (IQR) and categorical data were presented as frequencies with percentages. The Mann-Whitney U test was performed for continuous variables and Pearson's $\chi^2$ test was used for categorical variables. For all analyses, a two-tailed $p$ value < 0.05 was considered statistically significant. Data were analyzed using the Statistical Package for Social Sciences (SPSS), version 26.

## Results

*Description of Cohort*
As shown in Table 1, a total of 212 cases were included in this study. Most cases were of Caucasian ethnicity (156 cases, 73.6%). The mean age was 7.9 years with a median of 2 years (range 0–55).

Cleft Type
Cleft gene panel testing is most frequently performed in patients with CP (48.6%), followed by cases with CL/AP (36.3%) and CL/A (11.8%) (Tables 1, 2).

Prenatal History Assessment and Detection of Oral Cleft
In 44 cases (20.8%), presence of an oral cleft was detected on routine ultrasound examination at 20 weeks of pregnancy (Table 1). Data on possible teratogenic factors were available in 67% (140/212) of the cases. The most frequent teratogenic factor was smoking, reported in 16 cases (7.5%).

Family History
In 50 cases (23.6%), there was a positive family history for orofacial clefting (Tables 1, 2).

Associated Anomalies
Prior to genetic testing, in 162 out of 212 cases (76.4%), additional anomalies and/or dysmorphic features were reported. Some of these features are classified as common variations by Merks and colleagues and therefore not categorized as a minor anomaly [Merks et al., 2006].

The presence of associated major abnormalities in our CLA/P cohort was higher than that reported in a group of healthy Dutch school children (28.8% and 15.5%, respectively) [Merks et al., 2006, 2008]. Also, the presence of three or more minor anomalies was more frequent (27.8 and 8.3%,

**Table 1.** Patient description and clinical characteristics

| Characteristics | Total | Gene panel testing | | p value |
| --- | --- | --- | --- | --- |
| | | confirmed diagnosis | no diagnosis | |
| n, (%) | 212 | 24 (11.3) | 188 (88.7) | |
| Age, years | | | | 0.463 |
|    Mean±SD | 7.9±10.7 | 5.0±6.2 | 8.2±11.1 | |
|    Median (IQR) | 2.0 (9) | 2.0 (7) | 3.0 (9) | |
| Gender, n (%) | | | | 0.629 |
|    Male | 107 (50.5) | 11 (45.8) | 96 (51.1) | |
|    Female | 105 (49.5) | 13 (54.2) | 92 (48.9) | |
| Prenatal detection cleft by 20 weeks' ultrasound, n (%) | | | | 0.322 |
|    Yes | 44 (20.8) | 7 (29.2) | 37 (19.7) | |
|    No | 100 (47.2) | 8 (33.3) | 92 (48.9) | |
|    Unknown | 68 (32.1) | 9 (37.5) | 59 (31.4) | |
| Cleft type, n (%) | | | | 0.987 |
|    CL/A | 25 (11.8) | 3 (12.5) | 22 (11.7) | |
|    CL/AP | 77 (36.3) | 8 (33.3) | 69 (36.7) | |
|    CP | 103 (48.6) | 12 (50.0) | 91 (48.4) | |
|    NC | 7 (3.3) | 1 (4.2) | 6 (3.2) | |
| Consanguinity, n (%) | | | | 0.671 |
|    Yes | 6 (2.8) | 0 | 6 (3.2) | |
|    No | 173 (81.6) | 20 (83.3) | 153 (81.4) | |
|    Unknown | 33 (15.6) | 4 (16.7) | 29 (15.4) | |
| Family history positive for cleft-related disorders and/or congenital anomalies, n (%) | | | | 0.998 |
|    Yes | 107 (50.5) | 12 (50.0) | 95 (50.5) | |
|    No | 87 (41.0) | 10 (41.7) | 77 (41.0) | |
|    Unknown | 18 (8.5) | 2 (8.3) | 16 (8.5) | |
| Family history CLA/P, n (%) | | | | 0.397 |
|    Yes | 50 (23.6) | 4 (16.7) | 46 (24.5) | |
|    No | 162 (76.4) | 20 (83.3) | 142 (75.5) | |
| Minor anomalies, n (%) | | | | 0.177 |
|    Yes | 123 (58) | 17 (70.8) | 106 (56.4) | |
|    No | 89 (42) | 7 (29.2) | 82 (44.4) | |
|    Unknown | 0 | 0 | 0 | |
| Major anomalies, n (%) | | | | 0.070 |
|    Yes | 61 (28.8) | 11 (45.8) | 50 (26.6) | |
|    No | 143 (67.5) | 13 (54.2) | 130 (69.1) | |
|    Unknown | 8 (3.7) | 0 | 8 (4.3) | |
| >3 minor anomalies, n (%) | | | | 0.862 |
|    Yes | 59 (27.8) | 7 (29.2) | 52 (27.7) | |
|    No | 147 (69.3) | 17 (70.8) | 130 (69.1) | |
|    Unknown | 6 (2.8) | 0 | 6 (3.2) | |
| >3 minor and/or cleft syndrome-associated features, n (%) | | | | **0.032** |
|    Yes | 87 (41.0) | 15 (62.5) | 72 (38.3) | |
|    No | 119 (56.1) | 9 (37.5) | 110 (58.5) | |
|    Unknown | 6 (2.8) | 0 | 6 (3.2) | |
| Syndrome diagnosis considered pretesting, n (%) | | | | **0.001** |
|    Yes | 84 (39.6) | 18 (75.0) | 66 (35.1) | |
|    No | 120 (56.6) | 6 (25.0) | 114 (60.6) | |
|    Unknown | 8 (3.8) | 0 | 8 (4.3) | |

CL/A, cleft lip with or without alveolus; CL/AP, cleft lip with or without alveolus and cleft palate; CP, cleft palate only; CLA/P, clefts of the lip, alveolus and/or palate; NC, not classified.

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**Table 2.** Yield cleft gene panel testing according to cleft type, considered syndromic/non-syndromic cleft and familial cleft

| Characteristics | Total | Gene panel testing | |
|---|---|---|---|
| | | confirmed diagnosis | no diagnosis |
| Cleft type, *n* (%) | | | |
| CL/A | 25 (11.8) | 3 (12.5) | 22 (11.7) |
| CL/AP | 77 (36.3) | 8 (33.3) | 69 (36.7) |
| CP | 103 (48.6) | 12 (50) | 91 (48.4) |
| NC | 7 (3.3) | 1 (4.2) | 6 (3.2) |
| Total | **212 (100.0)** | **24 (11.3)** | **188 (88.7)** |
| Syndrome diagnosis considered pretesting, *n* (%) | | | |
| CL/A | 10 (11.9) | 2 (11.1) | 8 (12.1) |
| CL/AP | 28 (33.3) | 6 (33.3) | 22 (33.3) |
| CP | 42 (50) | 9 (50) | 33 (50) |
| NC | 4 (4.8) | 1 (5.6) | 3 (4.5) |
| Subtotal | **84 (39.6)** | **18 (75.0)** | **66 (35.1)** |
| Syndrome diagnosis *not* suspected pretesting, *n* (%) | | | |
| CL/A | 14 (11.7) | 1 (16.7) | 13 (11.4) |
| CL/AP | 47 (39.2) | 2 (33.3) | 45 (39.5) |
| CP | 56 (46.7) | 3 (50.0) | 53 (46.5) |
| NC | 3 (2.5) | 0 | 3 (2.6) |
| Subtotal | **120 (56.6)** | **6 (25.0)** | **114 (60.6)** |
| Syndrome diagnosis considering pretesting not defined | | | |
| CL/A | 1 (12.5) | 0 | 1 (12.5) |
| CL/AP | 2 (25.0) | 0 | 2 (25.0) |
| CP | 5 (62.5) | 0 | 5 (62.5) |
| NC | 0 | 0 | 0 |
| Subtotal | **8 (3.8)** | **0** | **8 (4.3)** |
| Family history of CLA/P, *n* (%) | | | |
| CL/A | 9 (18.0) | 0 | 9 (19.6) |
| CL/AP | 24 (48.0) | 3 (75.0) | 21 (45.6) |
| CP | 16 (32.0) | 1 (25.0) | 15 (32.6) |
| NC | 1 (2.0) | 0 | 1 (2.2) |
| Subtotal | **50 (23.6)** | **4 (16.7)** | **46 (24.5)** |
| No positive family history of CLA/P, *n* (%) | | | |
| CL/A | 16 (9.9) | 3 (15.0) | 13 (0.7) |
| CL/AP | 53 (32.7) | 5 (25.0) | 48 (33.8) |
| CP | 87 (53.7) | 11 (55.0) | 76 (53.5) |
| NC | 6 (3.7) | 1 (5.0) | 5 (3.5) |
| Subtotal | **162 (76.4)** | **20 (83.3)** | **142 (75.5)** |

CL/A, cleft lip with or without alveolus; CL/AP, cleft lip with or without alveolus, and cleft palate; CP, cleft palate only; CLA/P, clefts of the lip, alveolus and/or palate, NC, not classified.

respectively). The frequency of observed minor anomalies was comparable (58% and 65.1%, respectively).

In 41.0% of the cases (*n* = 87), three or more minor anomalies and/or a specific cleft syndrome-related feature was present (Table 1). Specific cleft syndrome-related features were present in 18 cases (online suppl. Table S1). Mental retardation (2.4%), neurologic abnormalities (3.3%), gastrointestinal problems (1.4%), and growth retardation were seen in a minority of cases (1.4%).

*Genetic Testing and Confirmed Syndrome Diagnoses*
Gene Panel Testing

Cleft gene panel testing in the 212 cases yielded a molecular diagnosis in 24 cases (11.3%); single nucleotide pathogenic gene variants were found in 23 cases and a complete gene deletion (*MEIS2* gene) in one case. A broad spectrum of diagnoses was identified (Table 3). The yield (*n* = 4) in the 50 cases with a positive family history for CLA/P the yield was 8% (Tables 1, 2).

**Table 3.** Pathogenic gene variants (class 5, pathogenic) and CNV in cleft-related genes by WES-based cleft gene panel analyses

| Gene | Variant | Syndrome | Cleft type |
|---|---|---|---|
| **SMAD4** (NM_005359.5) | c.[1486C>T];[=] p.[(Arg496Cys)];[(=)] | Myhre syndrome (OMIM # 139210) | CL/A |
| **TFAP2A** (NM_003220.2) | c.[710G>C];[=] p.(Arg237Pro) | Branchiooculofacial syndrome (BOFS) (OMIM # 113620) | CL/A |
| **MSX1** (NM_002448.3) | c.[901dup];[=] p.[(His301fs)]; [(=)] dn | Tooth agenesis, selective, 1, with or without orofacial cleft (OMIM # 106600) | CL/A |
| **CTNND1** (NM_001085458.1) | c.[1595G>A];[=] p.[(Gly532Asp)];[(=)] | Blepharocheilodontic syndrome 2 (BCDS2) (OMIM # 617681) | CL/AP |
| **CTNND1** (NM_001085458.1) | c.[1381C>T];[=] p.[(Arg461*)];[(=)] | Blepharocheilodontic syndrome 2 (BCDS2) (OMIM # 617681) | CL/AP |
| **SMAD4** (NM_005359.5) | c.[1498A>G];[=] p.[(Ile500Val)];[(=)] | Myhre syndrome (OMIM # 139210) | CL/AP |
| **SIX3** (NM_005413.3) | c.[546_562del];[=] p.[(Arg183fs)];[(=)] | Holoprosencephaly 2 (OMIM # 157170) | CL/AP |
| **MSX1** (NM_002448.3) | c.[605G>A];[=] p.[(Arg202His)];[(=)] | Orofacial cleft 5 (OMIM # 608874)/Tooth agenesis, selective, 1, with or without orofacial cleft (OMIM # 106600) | CL/AP |
| **LRP6** (NM_002336.2) | c.[3397+1G>A];[=] p.[(?)] | Tooth agenesis, selective, 7 (OMIM # 616724) | CL/AP |
| **IRF6** (NM_006147.3) | c.[235T>C];[=] p.[(Trp79Arg)] | van der Woude syndrome (OMIM # 119300) | CL/AP |
| **CHD7** (NM_017780.2) | c.[6850C>T];[=] p.[(Arg2284*)] dn | CHARGE syndrome (OMIM # 214800) | CL/AP |
| **FOXC2** (NM_005251.2) | c.[798del];[=] p.[(Leu267fs)] | Lymphedema-distichiasis syndrome (OMIM # 153400) | CP |
| **FOXC2** (NM_005251.2) | c.[456del];[=] p.[(Tyr153fs)] | Lymphedema-distichiasis syndrome (OMIM # 153400) | CP |
| **ZIC2** (NM_007129.3) | c.[1377_1385del];[=] p.[(Ala468_A470del)];[(=)] | Holoprosencephaly 5 (OMIM # 609637) | CP |
| **SMAD3** (NM_005902.3) | c.[221G>A];[=] p.[(Arg74Gln)] | Loeys-Dietz syndrome 3 (OMIM # 613795) | CP |
| **COL2A1** (NM_001844.4) | c.[1931dup];[=] p.[(Gly645fs)] | Stickler syndrome (OMIM # 108300) | CP |
| **DHCR7** (NM_001360.2) | c.[964-1G>C];[765C>A] p.[(?)]; [(Phe255Leu)] | Smith-Lemli-Opitz syndrome (OMIM # 270400) | CP |
| **GRHL3** (NM_198173.2) | c.[191_192del];[=] p.[(Tyr64*)] | van der Woude syndrome 2 (OMIM # 606713) | CP |
| **IRF6** (NM_006147.3) | c.[1198C>T];[=] p.[(Arg400Trp)];[(=)] | van der Woude syndrome (OMIM # 119300); Orofacial cleft 11 (OMIM # 600625) | CP |
| **KCNJ2** (NM_000891.2) | c.[224C>T];[=] p.[(Thr75Met)];[(=)] | Andersen cardiodysrhythmic periodic paralysis (OMIM # 170390) | CP |
| **BCOR** (NM_017745.5) | c.[254del];[=] p.[(Pro85fs)];[(=)] | Oculofaciocardiodental Syndrome (OMIM # 300166) | CP |
| **COL2A1** (NM_001844.4) | c.[2813del];[=] p.[(Pro938fs)];[(=)] | Stickler syndroom (OMIM # 108300) | CP |
| **TXNL4A** (NM_001303471.2) | c.[101A>G(;)-488_-455del] p.[(Tyr34Cys)(;)(?)] | Burn-McKeown syndrome (OMIM # 608572) | NC |
| **MEIS2 deletion**[a] | Chr15q14 (?_37,168,550) _(37,188,993_?) del[a] | Cleft palate, cardiac defects, and mental retardation (OMIM # 600987) | CP |

CL/A, cleft lip with or without alveolus; CL/AP, cleft lip with or without alveolus, and cleft palate; CP, cleft palate only; NC, not classified. [a]Detected with cleft gene panel testing.

In total, 67 variants of uncertain clinical significance (class 3) were identified in 54 cases (25.5%). Gene panel analysis revealed a single pathogenic allele for an autosomal recessive cleft syndrome in 29 cases (13.7%) (online suppl. Table S7).

*Additional Genetic Testing*
In 174 of the 212 cohort cases, the diagnostic workup included additional genetic testing. In total, 216 additional tests were reported, including SNP array analysis (165), single gene analysis (10), other gene panel testing

**Table 4.** Chromosomal microdeletion or duplication by SNP array analysis (*n* = 165)

| SNP array | Variant | Cleft type |
|---|---|---|
| 1q24.2q24.3 (169,580,885–171,295,650) ×1 mat, 4q13.1q21.22 (61,404,788–83,218,230) ×1dn,4q24q28.1 (106,408,926–123,814,900) ×3 dn arr [hg19] | Deletion 4q13 (de novo) | CL/AP |
| 16p11.2 (29595483_30198151) ×1 mat arr [GRCh37] | 16p11.2 deletion (16p11.2 deletion syndrome OMIM # 611913) | CP |
| 16p11.2 (29,595,483–30,198,151) ×1 dn arr [hg19] | 16p11.2 deletion (16p11.2 deletion syndrome OMIM # 611913) | CP |
| 16q24.1 (84,737,619–84,822,855) ×1 mat,22q12.3 (33,645,415–33,737,634) ×3 pat[a] | 16q24.1 deletion (USP10) | CL/AP |

CL/A, cleft lip with or without alveolus; CL/AP, cleft lip with or without alveolus, and cleft palate; CP, cleft palate only. [a]In addition to de novo *ZFHX4* variant.

(20), open exome analysis (15), karyotyping (5), and FISH analysis 22q11.2 (1). In most cases (*n* = 128), cleft gene panel analysis was performed in combination with SNP array analysis (online suppl. Table S8). Four cases showed a copy number variant (CNV) by SNP array analyses (4/165) (Table 4; online suppl. Table S8).

In 5 cases, a pathogenic gene variant was identified by the following tests (5/41): Sanger sequencing of the *ANKRD11* gene (*n* = 1), WES-based craniofacial gene panel analyses l (*n* = 1), WES-based targeting of 3024 OMIM morbid genes (*n* = 1), and trio WES (*n* = 2) (Table 5; online suppl. Table S8). The variant in *ARHGAP29* (NM_001328664.1) was not detected by gene panel testing because this gene was not yet included in the applied version of the cleft gene panel.

Karyotyping (*n* = 5) and FISH analysis 22q11.2 (*n* = 1) performed in addition to cleft gene panel testing did not reveal a chromosomal anomaly. Additional testing, performed in 174 out of 212 cases, revealed a pathogenic gene variant and/or CNV in eight cases, corresponding to 3.8% (8/212) of the total cohort (Tables 4, 5).

In one of these cases, a pathogenic variant in the novel cleft gene *ZFHX4* gene with a concurrent deletion 16q24.1 deletion, encompassing the gene *USP10*, was identified. This case is recently reported as a separate case report [Créton et al., 2023]. Inclusion of the results of the reported additional tests would increase the total yield of confirmed diagnoses in this cohort to 32 of 212 cases (15.1%).

*Correlation of Confirmed Diagnoses to Cleft Type*

The different cleft types (CL/A, CL/AP, and CP) did not reveal a significant difference in diagnostic yield (Pearson $\chi^2$ test, *p* = 0.987) (Table 1). In two out of the three CL(A) cases, a cardinal syndromic feature (BOF syndrome; OMIM # 113620 and MSX1-related orofacial clefting/STHAG1; OMIM # 106600) was indicative for the underlying syndrome (OMIM # 106600) (online suppl. Table S9).

*Correlation of Confirmed Diagnoses and Associated Anomalies*

In the 24 CLA/P cases with a confirmed molecular diagnosis provided by gene panel testing, the suspicion of an underlying genetic diagnosis prior to testing was significantly higher (*p* = 0.001) in comparison to the CLA/P cases without a molecular diagnosis (Table 1). Also, the presence of 3 or more minor anomalies and/or a striking cleft syndrome-related feature was significantly more prevalent (*p* = 0.032) (Table 1).

In five of the 24 cases (20.8%), additional anomalies were noted after identification of the underlying genetic diagnosis. In three of 18 of the 24 CLA/P cases with a suspected syndrome diagnosis prior to testing, the specific diagnosis could be confirmed (van der Woude syndrome, MSX1, Stickler syndrome). For the remaining cases, the molecular diagnosis was not recognized prior to gene panel testing.

For example, the CL/A case, with an identified pathogenic variant in *SMAD4* (Myhre syndrome; OMIM# 139210), showed only a patent ductus arteriosus (HP: 0001643). The family history was negative and did not suggest an underlying genetic cause (online suppl. Table S9).

In the CL/AP neonate, with a pathogenic *CDH7* variant, CHARGE syndrome was initially not recognized due to the presence of striking non-characteristic hypertelorism (HP:0000316) and absence of characteristic abnormality of the pinna (HP:0000377) (online suppl.

**Table 5.** Pathogenic variants in cleft-related genes by additional genetic testing (single gene analyses, additional gene panel analyses, trio WES analyses) (*n* = 41)

| Gene test | Gene | Variant | Class | Syndrome | cleft type |
|-----------|------|---------|-------|----------|------------|
| Trio WES | *ZFHX4* [a] (NM_024721.4) | c.[2513del];[=] p.[(Asn838fs)] | 5 (P) | | CL/AP |
| Trio WES | *MAPRE2* (NM_014268.3) | c.[172A>G];[=] p.[(Met58Val)] | 5 (P) | Symmetric circumferential skin creases, congenital, 2 (OMIM # 616734) | CP |
| Genepanel analyses Craniofacial anomalies* (151 genes) | *ARHGAP29*[b] (NM_001328664.1) | c.[955—8C>A];[=] (r.spl?)] | 5 (P) | ARHGAP29 associated nonsyndromic cleft lip with or without cleft palate (PMID: 28029220; PMID: 32698641) | CL/AP |
| Gene panel analyses Mendeliome** (3,605 genes) | *BMP2* | | 5 (P) | Short stature, facial dysmorphism, and skeletal anomalies with or without cardiac anomalies (OMIM # 617877) | CP |
| Single gene analyses | *ANKRD11* (NM_001256182.1) | c.[3123_3126del];[=] p.[(Ile1042fs)] | 5 (P) | KBG syndrome (OMIM # 148050) | NC |

Trio WES, whole exome sequencing in trio; NC, not classified. *https://www.radboudumc.nl/getmedia/46763550-fa16-4bac-a1dc-4c081b583cf5/CRANIOFACIAL-ANOMALIES_DG214.aspx (Mendelian inherited disorder – 3,605 genes; also see Table S9 supplementary data). **https://www.radboudumc.nl/getmedia/1dd6b509-65a9-4b80-a78d-39d8ae6b829e/MENDELIOME-GENE-PANEL_DG214.aspx (craniofacial anomalies – 151 genes; also see Table S10 supplementary data). [a]In addition to 16q24.1 deletion. [b]At later stage included in cleft gene panel.

Table S9). Similarly, no striking suggestive features of the identified diagnosis before testing were present in the CL/AP cases with blepharocheilodontic syndrome 2; the CLAP case with tooth agenesis, selective, type 7; and the CP cases with Van der Woude syndrome, type 1 and type 2 (online suppl. Table S9).

In the CP case with lymphedema-distichiasis syndrome (OMIM # 153400), the diagnosis could only be confirmed after additional ophthalmologic investigation, additional segregation analyses in the mother and grandfather, and clinical evaluation in the mother. In the cases with van der Woude syndrome, both types (OMIM # 119300 and OMIM # 606713) could only be distinguished by gene panel testing. In four (16.7%) out of the 24 cases with a detected pathogenic variant (*CTNND1, COL2A1, DHCR7, TXNL4A*), neither associated anomalies nor the family history led to the underlying cause before genetic testing (online suppl. Table S9).

### Confirmed Diagnosis Related to Prenatal Detection of CLA/P

In 7 of the 44 already antenatally identified CLA/P cases (29.2%), postnatally the following diagnosis were confirmed: Myhre syndrome, holoprosencephaly type 2, orofacial cleft type 5, CHARGE syndrome, Van der Woude syndrome, BCD syndrome type 2, and BOF syndrome. In these cases, a syndrome diagnosis was already suspected after birth, based on additional malformations and/or family history (online suppl. Table S9).

In 4 of these 7 cases (6 CLP, 1 CLA), additional major anomalies were reported. No data on additional anomalies identified prenatally are available.

### Influence of the Outcome of Genetic Testing on Treatment Options

Most of the molecular diagnoses (83%, 20/24) led to early and timely follow-up and tailored management. For example, identification of a class 5 pathogenic *KCNJ2* variant (Andersen-Tawil syndrome) led to cardiac follow-up in a CP patient and his father, who carried the same variant, revealing a cardiac arrhythmia phenotype.

### Discussion

In this study, diagnostic cleft gene panel testing has identified a molecular diagnosis in 24 out of 212 CLA/P cases (11.3%) in both suspected syndromic cleft (*n* = 18) and presumed non-syndromic cleft cases (*n* = 6). For familial cases (*n* = 50), the diagnostic yield was 8% (*n* = 4).

The total diagnostic yield of 15.1% in this study demonstrates that genetic testing, particularly WES-based CLA/P curated gene panels, is a powerful diagnostic tool in the diagnostic workup of CLA/P in addition to clinical assessment, leading to tailored monitoring and

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follow-up. Especially, significant Mendelian diagnosis is identified by cleft gene panel testing in apparently isolated and/or non-familial cases. Although, in these cases, the cleft is mainly regarded as a congenital malformation with a multifactorial inheritance pattern.

The yield of 8% in CLA/P cases ($n = 4$), with a positive family history for oral cleft, suggests that a complex etiology might play a role as well in familial cleft cases. However, pathogenic gene variants in novel cleft gene(s), not yet identified, or non-coding regions still might have been missed. Basha and colleagues identified pathogenic gene variants in four genes known to be mutated in CL/P, in 10% ($n = 5$) of 46 CLA/P, IRF6-negative, index cases with a family history of non-syndromic clefts of the lip and/or cleft palate. In these index cases, WES was performed [Basha et al., 2018].

In our study, cleft gene panel testing displayed a wide spectrum of underlying diagnoses. The most frequent diagnosis was Van der Woude syndrome ($n = 3$), followed by Stickler syndrome, blepharocheilodontic syndrome 2, Myhre syndrome, lymphedema-distichiasis syndrome, and MSX1-related orofacial clefting ($n = 2$ per syndrome). Interestingly, 22q11.2 DS was not identified in this CLA/P cohort, even though UMCU is an ERN-Cranio expertise center for 22q11.2 DS (ERN-Cranio). We hypothesize that in these cases, an SNP array is often performed in an early stage, confirming the 22q11.2 deletion, making a cleft gene panel superfluous, and therefore this group might not be present in our gene panel cohort.

In cases with a confirmed diagnosis, a syndrome diagnosis was significantly more frequently suspected by the clinical geneticist prior to testing, reflecting the importance of clinical assessment in the diagnostic workup. However, in 6 out of the 24 cases with a molecular diagnosis identified by cleft gene panel testing, prior to testing, no underlying syndrome diagnosis was suspected. Furthermore, only in 3 cases, the exact underlying syndrome was recognized by the clinical geneticist: (1) van der Woude syndrome, (2) STHAG1, and (3) Stickler syndrome. By genetic testing, both types of Van der Woude (type 1 [OMIM # 119300] and type 2 [OMIM # 606713]) could be distinguished. This illustrates the importance of NGS-based gene panel testing in CLA/P patients. In clinical practice, a syndrome can easily be missed due to the absence of known characteristic features or the wide clinical variability of the syndrome. Also, some of these features require additional evaluation or will appear only later in life [Rittler et al., 2011]. In almost one-third of antenatally identified CLA/P cases, a genetic diagnosis was made

postnatally by cleft gene panel testing. This underscores the relevance of offering gene panel testing for expecting parents in order to ensure informed decision-making in pregnancy.

Since cleft gene panel testing can reveal clinically unrecognized genetic diagnoses, one might consider mainstreaming as a possible benefit of this testing. Integrating genetic testing into the general practice of nurses and physicians, without expertise in cleft syndrome diagnostics, might reduce health care costs. However, this study also demonstrates the significance of integrated genetic and clinical evaluation in the etiologic diagnostics. In some cases, tailored clinical evaluation based on the outcome of cleft gene panel testing resulted in a confirmed molecular diagnosis. Additionally, in some cases, clinical and segregation analyses of patients and family members further confirmed the diagnosis (e.g., lymphedema-distichiasis syndrome [OMIM # 153400] and Andersen cardiodysrhythmic periodic paralysis [OMIM # 170390]). This illustrates the theorem of Hennekam and Biesecker: "diagnostic skills of medical specialists will shift from a pre-NGS-test differential diagnostic mode to a post-NGS-test diagnostic assessment mode" [Hennekam and Biesecker, 2012]. Finally, gene panel and broader genetic testing regularly leads to the identification of variants of unknown significance (VUS) which require interpretation by highly skilled professionals in this field before the results are discussed with the patient and family.

Recently, Lustosa-Mendes and colleagues reported on the predictive value of the presence of additional minor anomalies in diagnostics of a large cleft cohort; the mean number of minor signs in their oral cleft cohort was statistically higher in cases with abnormal chromosomal microanalysis results [Lustosa-Mendes et al., 2021]. This phenomenon was also described for other disorders, e.g., autism [van Daalen et al., 2011]. In our study, the percentage of three or more minor anomalies did not differ significantly between the cases with and without a confirmed molecular diagnosis (29.2% vs. 27.7%, respectively), suggesting that the presence of minor anomalies is not predictive for a molecular diagnosis in contrast to CNVs.

We did find a higher percentage of associated anomalies (76.4%) in our CLA/P cohort than reported in previous studies (range 3%–63%) [Lustosa-Mendes et al., 2021]. This wide range in percentages can be caused by differences in study population, including cleft type and age, or to varying definitions of anomalies and study design.

In line with previous studies, most diagnoses were made in CP cases [Maarse et al., 2012]. However, the molecular diagnostic yield was not significantly different between the different cleft types (CL/A, CL/AP, and CP) ($p = 0.987$). In this respect, it is important to realize that our cohort reflects a certain bias in which CP is over-represented. The Dutch national registry, recording 3,512 patients with a common oral cleft from 1997 to 2006, showed 33% of all Dutch CLA/P patients have a CP, 39% a CL/AP, and 28% exhibited CL/A [Luijsterburg et al., 2014], while in our study CP was present in almost half of all cases (48.6%) followed by CL/AP (36.8%) and CL/A (11.8%). Furthermore, in two out of three CL/A cases with a confirmed genetic diagnosis, the underlying syndrome diagnosis was suspected prior to testing. This also reflects a certain bias.

In addition to the value of cleft gene panel testing in the diagnostic workup of CLA/P, this study demonstrates the benefit of broader genetic testing in cleft cases without a diagnosis by gene panel testing only. In some very specific cases, a molecular diagnosis was identified by additional genetic testing, including additional gene panels or WES and SNP array. Interestingly, in a girl with a unilateral CLA/P, WES revealed a *de novo* pathogenic variant in the novel cleft gene *ZFHX4* concurrently with a 16q24.1 deletion, encompassing the gene *USP10*. This case is reported as a separate case report, supporting *ZFHX4* as a novel cleft gene and demonstrating co-occurrence of a pathogenic gene variant and a chromosome deletion, and may contribute to the etiology of orofacial cleft (Créton. 2023).

Although this study provides unique knowledge on outcomes of cleft gene panel testing and additional genetic testing in children with several cleft types, we realize this study has some important limitations. This retrospective inventory reflects the outcome of a biased population, often performed (39.6%) in cases suggestive for a possible underlying genetic cause. Furthermore, due to the retrospective nature of this study, the data were incomplete. Although all patients were clinically evaluated by an experienced clinical geneticist, phenotypic evaluation was not standardized and likely associated within inter-observer variations. Furthermore, there was no standardized follow-up.

Another limitation is the changing content of the diagnostic gene panels (genes were added each year) and additional broad genetic testing was not conducted in all cases. Implementation of novel technical platforms in genome diagnostics (e.g., WGS, long read sequencing) and their increasing implementation in clinical practice might lead to a higher diagnostic yield.

With these novel diagnostic methods, structural variations disrupting topologically associated domains (TADs), accountable for a number of skeletal and cranial developmental syndromes, can also be identified [Lupiáñez et al., 2016]. Such genomic variations will be missed by the currently performed diagnostic cleft gene panel testing (WES based) in this patient cohort.

With these limitations in mind, this study clearly highlights the benefit of cleft gene panel analysis in children born with an oral cleft. Cleft gene panel analysis leads to (1) early diagnosis of syndromes preventing a long diagnostic odyssey; (2) early identification of syndromes requiring specific monitoring and follow-up, especially in clinically unsuspected cases; (3) improvement of genetic counseling with a more tailored recurrence risk by identification of the underlying cause.

Considering the increasing change to earlier, even prenatal, diagnoses in cleft cases, we feel that it is important that future studies are initiated as to gain more insight into the psychosocial and clinical impact of these early diagnoses and how these impact the quality of life of cleft cases and their families.

In conclusion, this study illustrates the benefit of cleft gene panel testing in all types of oral clefts and demonstrates cleft gene panel testing as a powerful diagnostic tool, also in cases with an apparently isolated and/or non-familial oral cleft. It facilitates differentiating non-syndromic from syndromic oral cleft cases and provides for accurate recurrence risks. This study also demonstrates the power of integrating molecular testing with thorough clinical evaluation in order to reveal unrecognized underlying genetic diagnoses. In our opinion, all oral pre- and postnatal cleft cases should be referred to a clinical geneticist for detailed phenotyping, exploration of the family history, and genetic counseling on possibilities for genetic testing.

## Statement of Ethics

Ethical approval for this retrospective study and a waiver of informed consent was obtained from the UMCU Ethical Committee (local number: 19-061/c).

## Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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## Author Contributions

Marie-José Henriette van den Boogaard and Aebele Barber Mink van der Molen designed the study and helped write the manuscript. Lisca Florence Wurfbain performed the statistical analysis, analyzed the data, and helped write the manuscript. Inge Lucia Cox analyzed the data and helped write the manuscript. Maarten Pieter Gerrit Massink and Augusta Maria Antonia Lachmeijer helped write the

manuscript. Maria Francisca van Dooren, Virginie Johanna Maria Verhoeven, Johanna Maria van Hagen, Malou Heijligers, Jolien Sietske Klein Wassink-Ruiter, Saskia Koene, Saskia Mariska Maas, Hermine ElisabethVeenstra-Knol, and Johannes Kristian Ploos van Amstel helped by delivering essential data.

## Data Availability Statement

Genetic testing was part of the diagnostic workup in the clinic. The DNA analyses were performed after extensive genetic counseling and based on shared decision-making. The data on gene panel testing, where we report on, are partly derived from whole exome sequencing as part of clinical practice (as outlined above). While the data are obtained in clinical care, patients are not part of a research cohort or biobank and therefore did not give their consent to making their *complete genetic* data publicly available. However, we would welcome to show the data and demonstrate filtering processes/practice on site.

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