Genetic Investigations of Cleft Lip with or without Cleft Palate: A Literature Review and Genome-wide Association Study in a Filipino Population

by

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Genetic Investigations of Cleft Lip with or without Cleft Palate: A Literature Review and Genome-wide Association Study in a Filipino Population

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Cleft lip with or without cleft palate (CL/P) is one of the most common congenital anomalies worldwide, with a complex etiology involving both genetic and environmental factors. This thesis provides a comprehensive literature review of the epidemiology, psychology, healthcare costs, and genetics of CL/P, followed by an original genome-wide association study (GWAS) and downstream analyses in a Filipino population. The literature review traces the historical understanding of CL/P, discussing its epidemiology across different populations, the psychological impact on affected individuals, and the substantial healthcare costs associated with treatment. The review then discusses the genetic aspects of CL/P, covering heritability estimates, segregation analyses, linkage studies, genome-wide association studies (GWAS), gene-environment interactions, sequencing studies, animal models, and phenotypic heterogeneity. The original research presented is a GWAS of CL/P in a Filipino cohort of 1,399 unrelated individuals (882 cases, 517 controls). The study identified a novel locus, *KCNQ5*, and replicated associations at three known loci: *ARHGAP29*, *IRF6*, and *SHROOM3*. A gene-based test using mBAT-combo highlighted potential masking effects in several genes, including *IRF6*. A transcriptome-wide association study (TWAS) further supported the involvement of known clefting loci. Fine-mapping analyses and conditional and joint multiple-SNP analysis (COJO) provided insights into putative causal variants, particularly the missense variant rs2235371 in *IRF6*, which showed strong functional evidence and high linkage disequilibrium with the top signal. This thesis underscores the importance of conducting genetic studies in diverse populations to better understand the genetic architecture of complex traits like CL/P and to address disparities in genetic research. The public health relevance of this work lies in its potential to improve our understanding of CL/P etiology, which could lead to better prevention strategies, earlier diagnosis, and more targeted treatments, ultimately reducing the burden of this condition on affected individuals, families,

and healthcare systems. The findings contribute to the growing body of knowledge on the genetic basis of CL/P and provide a foundation for future research aimed at elucidating the etiology of this condition and improving strategies for prevention, diagnosis, and treatment.

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Preface

This thesis is dedicated to my sister (Grace) and my mother (Mom). Your love and support has meant the world to me. I love you both very much.

I would also like to thank all the participants in the Philippines for contributing the DNA samples that made this thesis possible.

1.0 Literature Review

1.1 Introduction and a Word From the Author

Cleft lip with or without cleft palate (CL/P) is among the most common congenital anomalies worldwide. While treatment options exist, they are expensive and often limited to nations with relatively developed healthcare systems. Additionally, the burden borne by affected individuals, their family members, and societies is non-negligible. This literature review seeks to give a broad overview of what is currently known about CL/P and its various causes and effects.

In the process of writing this review, I have made some unconventional choices that warrant explanation. My aim was to provide a comprehensive and engaging overview of the subject matter while staying true to my own understanding and interpretation of the material. In certain sections, I have chosen to rely on the broadest meta-analyses available, operating under the assumption that the quality of information gleaned from such sources is often preferable to the disparate conclusions drawn from multiple individual studies. Similarly, in some instances, I have elected to dedicate more space to the in-depth exploration of a single, large-scale study that I deemed to be of exceptional quality or particularly thought-provoking, rather than simply listing the findings of numerous studies in succession.

At times, I have taken the liberty of delving into historical material, with the hope that readers might find these detours both enlightening and engaging. Additionally, I have occasionally stepped back from the purely objective reporting of facts to offer more subjective impressions of the field, adopting a somewhat more informal tone in the process. It is my hope that these strike the reader as enjoyable diversions rather than lapses in academic seriousness.

While I have strived to appropriately emphasize the most crucial aspects of the subject matter, the distribution of time and attention given to various topics should not be misconstrued as a precise reflection of their relative importance but rather–in accordance with whatever degree the reader considers charitability warranted–as the outcome of a student's

somewhat hurried engagement with the literature, a process unexpectedly set in motion by a series of unanticipated events that unfolded between the typical initiation of a thesis and its sooner-than-anticipated submission deadline.

Finally, there are instances where I have ventured slightly beyond the explicit boundaries of the subject matter in order to provide context that I believed might be beneficial for readers. The inclusion of such material is intended to enhance understanding and foster a more comprehensive appreciation of the topic at hand.

1.2 Epidemiology

The most frequently cited figure in the epidemiology of CL/P is Habib (1978). In turn citing Emery's Elements of Medical Genetics (1975), Habib reported a global incidence of 1 per 1000 live births, further broken down by racial variation as 0.6-1.7 for Caucasians, 0.4 for Blacks and 1.7 for Japanese. He also reported a sex bias for CL/P favoring males of between 0.6 and 0.8 and a sex bias favoring females for cleft palate (CP), an observation that has been consistently reported by subsequent studies.

A more recent study, Tanaka et al. (2012) sought to investigate these estimates as well as any potential changes over the intervening decades. Pooling data from the National Birth Defects Prevention Network, and the National Vital Statistics Reports from the Center for Disease Control (CDC) and reporting their estimates per 10,000 live births, they estimate an international prevalence of 7.94 (95% CI: 7.85-8.03) (equivalent to roughly 0.8 per 1000). In line with Habib's estimate, they found the highest national incidence to be that of Japan (19.05). Additionally, they reported a slight decrease of international incidence, although this was not statistically significant, possibly owing to the small number of years analyzed. Aggregating data within continents, they found prevalence rates for Asia, the Americas, and Europe of 10.2, 7.81, and 6.61 respectively. Although Africa was excluded from the continental analysis due to lack of data, they were able to estimate incidence for the country of South Africa, which was 3.13, the lowest recorded among all countries included in the country-level analysis, also in line with Habib's observations.

Recently, several studies have provided prevalence estimates and assessed lifestyle and demographic risk factors associated with CL/P in the United States. Heydari et al. (2024) performed a cross-sectional population based retrospective study using data from the National Center for Health Statistics (NCHS) and the CDC. They reported the prevalence per 10,000 live births between 2016 and 2021 as 4.88 (4.79-4.97) as well as estimates for males 5.96 (5.82-6.10) and females 3.75 (3.64-3.87). These estimates provide the male to female ratio of $5.96/3.75 = 1.59$. Taking the lower end of Habib's estimates (0.6) yields a male to female ratio of $0.6/0.4 = 1.5$, showing the two estimates are largely in agreement, despite the nearly half-century elapsed between them. Additionally, they identified several maternal risk factors for CL/P including younger age, obesity, use of assisted reproductive technology, pre-pregnancy hypertension and diabetes, and smoking–although with this last risk factor come the caveats that the prevalence rate reached its peak at 11-20 cigarettes a day before declining slightly at > 20 cigarettes a day (which they suggest might be an artifact of unmodeled confounding variables) along with increasing for all smoking groups over the time period studied.

Extensive research has been conducted to investigate the potential link between maternal smoking during pregnancy and the increased risk of cleft lip/palate (CL/P) in newborns, as smoking has been associated with numerous adverse health outcomes, particularly for children exposed to tobacco smoke in utero. Fell et al. (2022) conducted a systematic review and meta-analysis on this relationship using studies queried from Medline, Embase, Web of Science, and the Cochrane Library from inception to November 2020. While there had been previous comprehensive meta-analyses investigating this question, the authors raise concerns regarding the quality of the studies included. To remedy this, they used the Newcastle Ottawa Scale to interrogate various potential biases in the literature. Their analysis included cohort, case-control, quasiexperimental, natural experiment, family based negative control, and Mendelian randomization studies, and excluded conference proceedings, descriptive studies, reviews, animal studies, or studies with insufficient data to estimate effect sizes or for which full text was unavailable. They identified the most common problem as failure to adjust for four confounding factors, that they determined to be sufficiently justified: maternal age, maternal alcohol consumption, folic acid supplementation, and obesity. In

their discussion, they note that just three studies out of the 45 included in the review were judged to be of good quality. Their analysis suggested a moderate association between maternal smoking and orofacial cleft but highlighted the need for high quality research to further elucidate this relationship.

Tarista et al. (2024) reported an analysis of data from The National Birth Defect Prevention Network to estimate incidence differences across self-reported races. Stratifying their data by four year increments (2006-2010, 2010-2014, 2014-2018) and using the classifications of non-Hispanic White, non-Hispanic Black, Hispanic, Asian or Pacific Islander, and Native American or Alaskan Native, they found that Native American/Alaskan Natives were at highest risk, non-Hispanic Black and Asian/Pacific Islander were at lowest risk, and Hispanic and non-Hispanic Whites at an intermediate risk. Overall, they report national prevalence rates (per 1000 births) for these time increments of 0.78, 0.79, and 0.72, while noting that the decrease was not significant.

1.3 Psychology

In addition to epidemiological and etiological studies of CL/P, many investigators have also sought to assess the psychological impact of this condition on affected individuals.

Branson et al. (2024) conducted a systematic review and meta-analysis to determine if children born with CL/P were at an increased risk of psychological and peer difficulties. They note that previous literature and systematic reviews (e.g., Hunt et al., 2005) had not found conclusive evidence linking the two. English-language studies were queried from PsychINFO, EMBASE, and MEDLINE for the time period of January 2005 to January 2022 with the primary inclusion criterion being studies assessing children up to 17 years of age with CL/P that included measures of psychological outcomes. The data assessed came from 16 different countries with the majority coming from the USA, the UK, and Norway. Random effects meta-analysis was performed to account for the heterogeneity among different control populations. The authors report the only significant difference observed from self-reports as the decrease of conduct problems among affected children, but note that parent reports

reflected increases among affected children for emotional problems, conduct problems, and hyperactivity, albeit with relatively small effect sizes (Hedge's g: 0.25, 0.05, and 0.06, respectively) implying unlikely differences on a clinical level. Of note among their discussion were a few gender differences: girls reported more psychological symptoms than boys compared to control populations. Cleft visibility was associated with poorer social acceptance and fewer close friendships in girls, while this association was not observed in boys. More broadly, increased incidence of psychological symptoms was noted in children with visible clefts. Both treatment and its timing were found to have a positive impact on interpersonal relationships and self-esteem. The authors acknowledge the presence of conflicting evidence linking CL/P with psychological symptoms and conclude by echoing previous systematic reviews in calling for increased focus on study quality in order to more accurately assess this relationship.

1.4 Healthcare Costs

In addition to the psychological impact of CL/P, it is important to consider the substantial healthcare costs associated with its management and treatment. CL/P often requires a multidisciplinary approach, involving multiple surgeries, dental interventions, speech therapy, and long-term follow-up care, which can place a significant financial burden on families and healthcare systems alike.

In an attempt to assess recent trends in the costs borne by affected individuals and their families, Rochlin et al. (2022) queried the IBM MarketScan Commercial Database to extract information on patients younger than 18 who underwent primary or secondary CL/P repair between the years 2007 and 2016, totaling 6268 cleft lip and 9118 cleft palate repair episodes. They report that beneficiary contributions increased significantly over this time period (2006 medians: \$155.75 and \$124.37; 2017 medians: \$193.31 and \$183.22 for cleft lip and palate repair, respectively), largely attributed to an increase in deductibles. Additionally, they note a contrast of higher patient cost sharing geographically, with the increase in the South being more pronounced.

1.5 Genetics

As discussed above, the role of genetics in the etiology of CL/P, while still incompletely understood, is generally recognized as substantial. The progress of genetics research in the past couple of decades has been facilitated by the development and subsequent rapid drop in price of high-throughput genotyping and sequencing technologies, allowing researchers to investigate the more fine-grained details of the genetic contribution to various phenotypes, with CL/P being a prime example. Various methods have been used to interrogate the genetic component to CL/P, among the most prominent have been segregation analyses, linkage studies, candidate gene studies, GWAS, and sequencing studies. As the literature contains several excellent reviews outlining the progress and discoveries made in CL/P genetics, some of them quite recent (Leslie & Marazita, 2013; Watkins et al., 2014; Leslie & Marazita, 2015; Beaty et al., 2016; Nasreddine et al., 2021; Moreno Uribe & Marazita, 2023), we will seek to synthesize these findings, with an emphasis on recent trends and developments.

1.5.1 Heritability

CL/P is generally recognized to have a complex etiological origin, including a substantial genetic component. A common way this is assessed is through the estimation of heritability–that is, the proportion of phenotypic variance (in a given population, at a given time) attributable to genetic variation. While heritability can be estimated using molecular genetic data, the accuracy of these estimates are bound (often to considerable degree) by the genetic architecture of the trait in question. The majority of the data on genetic variation presently come from genome-wide association studies (GWAS), which are generally designed for assaying only common genetic variants (or variants highly correlated with them, which for statistical reasons, are generally common as well). If the contribution to the phenotypic variance of a trait comes from rare variants (as seems likely to be the case CL/P), estimates derived from molecular data are likely to be biased downwards as well as relatively imprecise. An alternative method for estimating heritability, which predates the use of molecular data, is through the use of expected genetic sharing of family members–often twins.

The largest study to date on CL/P in twins was conducted in 2011 by Grosen et al., using data from a Danish national population-based cohort. The stated motivation for this study came from the observation that dizygotic (DZ) twins exhibited a greater risk of clefting than siblings, which indicated the possibility of intrauterine environmental factors contributing to this risk, suggesting the possibility that twinning may be a risk factor in CL/P. While the authors were unable to demonstrate support for this hypothesis, the considerable sample size ($n \approx 4.9M$) allowed them to provide many estimates of utility in understanding the genetic factors involved in CL/P. Among these were prevalences of oral cleft for twins and singletons (15.8 and 16.6 per 10,000, respectively; prevalence ratio = 0.95 (0.83-1.1)), concordance rates for CL/P among monozygotic and dizygotic twins (50% and 8%, respectively), and heritability estimates for CL/P and CP (91% and 90%, respectively). The heritability estimates were arrived at by fitting several variance components models and assessing model fit by Aikake's Information Criteria (AIC). The best fitting model was determined to be the AE model, suggesting contributions from additive genetic variance (A) and nonshared environment (E)–the remaining 9% and 10% for CL/P and CP, respectively. The authors additionally noted the greater than four-fold increase concordance for MZ twins vs DZ twins implied the likelihood of CL/P being polygenic–a conclusion that subsequent genetic studies have borne out and is generally recognized by cleft researchers today (Cheng et al., 2023). A few caveats warrant mentioning in interpreting these results. Firstly, zygosity was determined by a 4-item questionnare about physical resemblance. The authors note both that this method had been previously shown (Christiansen et al., 2003) to have a misclassification rate of less than 5%, but concede that this method might perform suboptimally when used to assess a phenotype such as a physical malformation. To gauge the possibility of this skewing the results, they used blood, serum, and enzyme determinants to verify the zygosity determination provided by the questionnaire for two subsets of their data and in these, also found a misclassification rate of less than 5%. An additional possibility the authors mention is the potential for nonpaternity, which would incorrectly classify half-siblings as full siblings.

While Grosen et al. (2011) provided valuable insights into the heritability of CL/P, a fuller understanding of the trait's etiology requires the identification (and ideally, subsequent functional analysis) of the specific implicated genetic variants. In recent years, GWAS have

been instrumental in beginning to identify these. Building on these findings, Ludwig et al. (2017) performed a GWAS on CL/P and further determined, using GCTA analysis, that 31.2% (SE=8.5%) of the estimated variance for risk in CL/P was attributable to common variants (in Europeans), with 25.5% contributed by 24 previously documented risk loci. Combined with the heritability estimates provided by Grosen et al. (2011), this seems to indicate the potential for a large role of rare variants in CL/P, perhaps partly explaining the recent focus on sequencing studies, which allow the interrogation of genetic variation too rare to be detected by common SNP arrays.

1.5.2 Segregation Analysis

Early genetic studies were primarily concerned with investigating the mode of inheritance of CL/P rather than identifying implicated loci. While determining the mode of inheritance is often a simple matter that can be resolved by the analysis of pedigree structures, in retrospect CL/P seems almost a condition designed to confound investigators. While showing obvious familial aggregation, it does not follow simple Mendelian patterns. Additionally, as outlined previously, it also shows ancestry and sex-specific differences, some of which do not even agree in the direction of association (e.g. cleft lip is more common in males, while cleft palate is more common in females).

1.5.2.1 Brief Historical Detour

The first description of the transmission patterns of orofacial clefting is generally considered to have been authored by German botanist, Christoph Jacob Trew (Trew, 1757). His "Observation", was first made available to the English-speaking public in 1993 when Alice Chabora and Sidney Horowitz published a translation by a member of the Columbia University Greek and Latin Department. Trew outlines three accounts of orofacial clefts: two individual cases and one of a family. The individuals are a skeleton of a small female infant, of which he provides an illustration of an "entirely missing palate" and an "otherwise quite healthy" girl, for whom he wonders about the possibility of constructing an instrument which might be fitted to assist her. The family he recounts is of a 40 year old workman, his

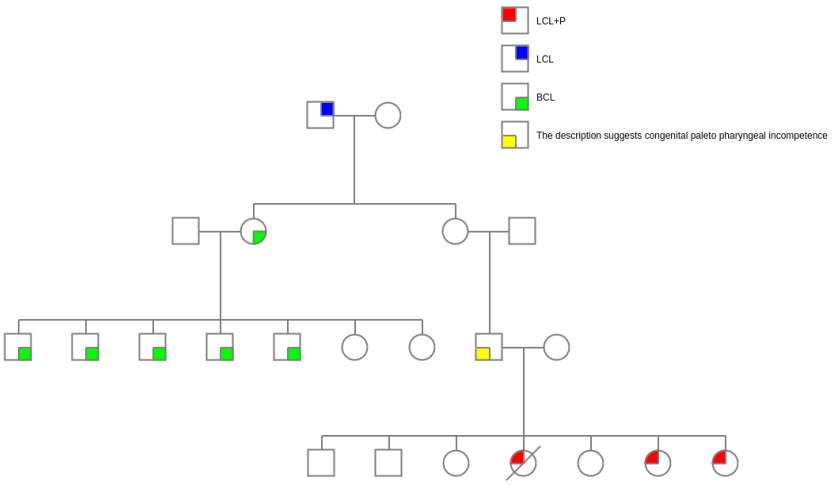

Figure 1: Pedigree diagram for the family described in the text of Trew's 1757 observation (Figure adapted from Chabora and Horowitz, 1974).

34 year old wife and their seven children (two boys and five girls). The two eldest children were both boys and both unaffected. Of the girls, the first and third were unaffected, while the second had both cleft lip and a missing palate (passing away at approximately eight months of age). The youngest two (at the time 15 months and 10 weeks respectively) are both reported as affected, yet "still rejoice in life." Trew goes on to note that while both parents were unaffected, the father's palate "appeared to be hardened and swollen, as if from some early defect which he knew nothing about." Today we might recognize this as an example of a "threshold case": one in which the two sides of the palate had successfully fused, but perhaps still show evidence of some developmental aberration. His maternal aunt, in turn, seems to present with cleft lip and gave birth to five affected sons and two unaffected daughters. Predating the revelations of the "replication crisis," Trew can be forgiven for not making his data public, although Chabora and Horowitz helpfully supply a pedigree reconstructed from the account.

Other early descriptions of CL/P include Sproule (1869), Darwin (1875), and Bateson (1909) and several excellent historical accounts are given of the investigation of CL/P (Marazita 2002 and 2012) and attempts at its treatment (Perko 1986 and Bhattacharya et

al. 2009).

Classic models of segregation analysis largely centered around the segregation ratio (the proportion of affected cases among a given mating type). While these offer the advantage of being analytically tractable, they are limited in scope to strict Mendelian inheritance and data from parent-offspring relationships. While it took some time for statistical software to catch up with the theoretical developments, more modern segregation analysis methods were eventually developed that allowed for the use of more complex (and hence in the case of CL/P, more realistic) inheritance patterns and family structures. Among the major classes of complex segregation analysis used in attempts to make inferences about the inheritance pattern of CL/P were the major locus transmission model, the mixed/unified model, and regression models (Marazita, 2002).

1.5.2.2 Interlude: The MFT

A point of some contention in the early literature regarded the status of the multifactorial threshold model (MFT), which held that the etiology of clefting was governed by an underlying liability, influenced by many genes of small effect in addition to environmental factors (originally posited by Falconer (1965), see also Curnow (1972), Curnow and Smith (1975), and Fraser (1976)). Early analyses [1] seemed to suggest that the data were consistent with MFT, (e.g. Woolf et al., 1964; Tanaka et al., 1969*; Fraser et al., 1970; Czeizel and Tusnady 1972; Koguchi 1975*; Carter et al., 1982; Hu et al., 1982), but these were largely descriptive in nature, leaving open the possibility that CL/P was under a different mode of inheritance.

Following these descriptive studies a wave of goodness-of-fit studies were published in the 1980s that sought to test the explicit predictions of the MFT. Melnick et al. (1980) using a sample of 1895 individuals from Denmark, concluded poor fit for both the MFT and a single-major locus and suggested an alternate model that posited an underlying homogeneous genetic risk modified by variable susceptibility to teratogens. Mendel et al. (1980)* found

[1] Author's note: I am relying heavily on a table summarizing these studies contained in Marazita (2002). As many of these studies are fairly old, I will be indicating with an asterisk (*) studies I have been unable to track down and confirm the conclusion of.

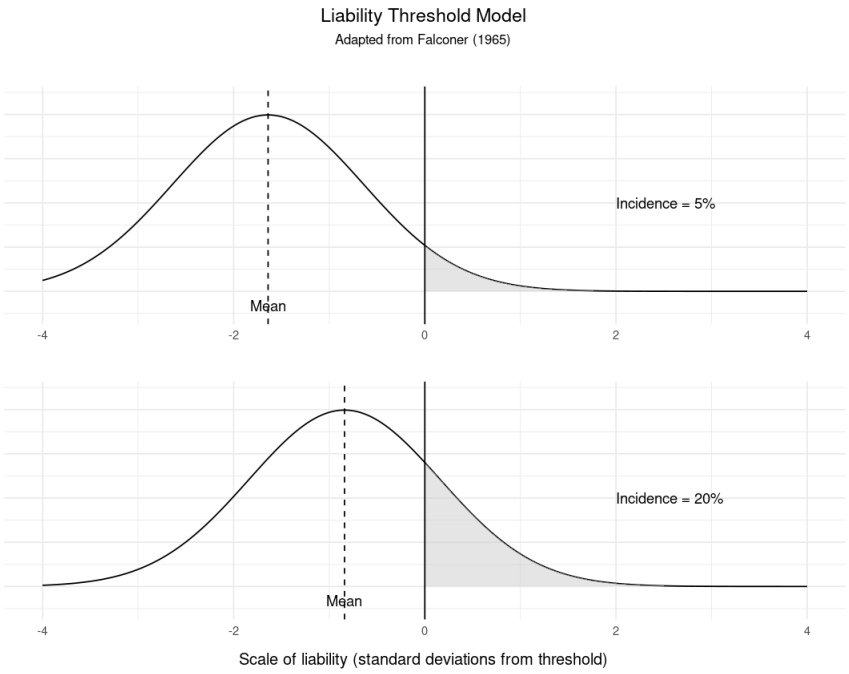

Figure 2: Illustrations of two populations or groups with different mean liabilities. The liability is normally distributed, with the same variance in the two groups. The groups are compared by reference to a fixed threshold. The stippled portions are the affected individuals with the incidences shown (Figure adapted from Falconer, 1965).

that the MFT fit in a certain region of the parameter space, but not all of it. Marazita et al. (1984) using a sample of 2,532 families (split into 26 multigenerational families with ≥ 4 affected family members and nuclear families with ≥ 2 affected children including a proband) from Denmark rejected the MFT in both samples. Marazita et al. (1986), using data from England, Denmark, and China rejected the MFT in all of these groups with the exception of some regions of the parameter space for the British subset. Nemana et al. (1992), using 331 proband families from India rejected the MFT model and suggested the data were most consistent with a major locus with reduced transmission probability, further suggesting a role for in utero exposure to harmful environmental agents.

One way investigators are able to interrogate the mode of inheritance of a heritable trait is through the application of segregation analysis. While originally used as a term for the assessment of strictly Mendelian patterns on inheritance, the term has become generalized to cases on a not-strictly Mendelian nature (sometimes called complex segregation analysis [CSA]).

Preceding and succeeding these goodness-of-fit studies were explicit segregation analyses that sought to determine which mode of inheritance was most consistent with a given dataset. Of the fifteen CL/P studies recounted in Marazita (2002), perhaps the strongest support was offered by Chung et al.,(1986) for which the MFT fit in the Japanse subset the best (although this was not the case in the Danish subset), Hecht et al. (1991), for which the MFT and recessive model fit equally well, and Clementi et al. (1995), where the MFT and major gene models fit equally well. For several studies, the results were ambiguous (e.g. Chung et al., 1974; Demenias et al., 1984; Marazita et al., 1984), and in several, the MFT was explicitly rejected in favor of another model: Marazita et al., 1986a, 1986b, 1992; Melnick et al., 1986; Chung et al., 1989; Nemana et al., 1992; Ray et al., 1993; Palomino et al., 1991,1997*; and Scapoli et al., 1999. Additionally reported are three of the previously mentioned studies which included subsets of only cleft palate, two of which were ambiguous (Chung et al. (1974) and Demenias et al. (1984)) and one rejected MFT (Clementi et al. (1997)).

As is often (some might argue, always) the case in science however, the data do not "speak for themselves" and there were challenges to the interpretations provided above. For example, Ray et al. (1993) rejected the MFT in favor of codominant or dominant model

inheritance based on Akaike's Information Criterion (AIC) values. In addition to the model fits, Ray et al. (1993) also report a sex ratio for simplex vs. multiplex families of 3.2 and 1.4, offering the explanation that "the loading of environmental factors predisposing to CLP is higher in multiplex families and results in a higher frequency of the less susceptible sex (females) becoming affected." Fraser (1998) however, offers a different—and admittedly more parsimonious—explanation that this is simply a result of different liability distributions (and thus thresholds) for the two sexes. That this lines up nicely with predictions made by the MFT, he argues is strong evidence for its validity. Another case of discrepant interpretations comes from a Letter to the Editor of the American Journal of Human Genetics in 1993, written by Ernest B. Hook, in response to the analysis published by Marazita et al. (1998). Hook raised two principal objections: one on statistical grounds and one on clinical grounds. The clinical implications will not be discussed here as 1) the aim of this review is to provide an overview of the statistical analyses and interpretations in the literature and 2) in their response, Marazita et al. (1993), seem to largely agree with Hook's points regarding clinical implications. Regarding the statistical disagreement, Hook argues that that while Marazita et al. (1998) report as the best-fitting model, the autosomal recessive major-locus model (as judged by AIC), models incorporating a multifactorial component fit the data better (as judged by the χ^2 values). The difference between these fit indices is essentially that while χ^2 values quantify a sort of "absolute" model fit, AIC, as mentioned above, quantify a model fit with an additional penalty term for extra parameters incorporated in the model (thus imposing sparsity). Hook suggests that while parsimony may be a desirable characteristic for statistical models, it need not follow that it accurately represents reality. In their response, Marazita et al. (1993) grant Hook's point about the AIC vs χ^2 values, but additionally note that the models he asserts "fit better" do not actually differ statistically. They explain that as models in their analysis were nested, the addition of parameters can only ever increase model fit (or leave it unchanged), and the meaningful question in this context is whether or not the increase in model fit was "statistically significant," which it was not.

In the years between the segregation analysis era and the genomics era however, further evidence was proffered that seemed to suggested the MFT was—at least to some approximation—substantiated. The majority of the studies recounted above had sample sizes in

the range of hundreds to low thousands. Combined with the limitations inherent in complex segregation analysis (Jarvik 1998), it was suggested by some that the most reasonable conclusion one could draw was that the results from CSA were inconclusive (Mitchell 1997). In 2010 however, Grosen et al. (2010) reported on a cohort of 6776 affected individuals and 54,229 relatives. This large dataset (The Danish Facial Cleft Database) allowed them to produce precise estimates for relatives of first, second, and third degree as well as recurrence risks by severity, specificity, and parent of origin effects. While, as noted above, these parameter estimates do not constitute a formal statistical test of the MFT, Grosen et al. (2010)'s results (e.g. higher recurrence risk among offspring and siblings compared to parents, specificity of clefting subtype, steep drop-off in recurrence from first to second and second to third degree relatives, and highest recurrence risk in least frequently affected sex—males for CP and females for CLP) provided strong evidence in support of the MFT.

Sussing out where the literature stands now in regards to the MFT is not entirely straightforward. To some degree, this appears to be a semantic matter. While some researchers make a distinction between a strict MFT ("multifactorial threshold model") and a model involving "genes of major effect with a multifactorial background," (e.g. Chung et al., 1989 & Marazita 2010) others tend to be more broad in their interpretation and refer simply to "multifactorial threshold models of inheritance" (e.g. Grosen et al., 2011). Nature is of course, indifferent to the discrete categories scientists impose on it, but for the purposes of gaining a general understanding as to the genetic character of CL/P, it may help here to think in terms of something like a spectrum with monogenic traits at one end and polygenic (or omnigenic (Boyle et al., 2017–but see Wray et al., 2018), if you prefer) at another. Generally speaking, the more polygenic a trait is, the smaller the effect sizes of the loci underlying it will be, hence the distinction sometimes made between "strict MFT" and "major loci with underlying factorial inheritance". (Here, the former is describing a model at the polygenic end of the spectrum whereas the latter shifted towards the monogenic end). Should the reader find cause to wonder if scientists are not just splitting hairs here, it should be remembered that these are matters of immediate consequence. Indeed for those in the business of genetic counseling, the assumed mode of inheritance for a given condition is indispensable information, possibly implying entirely different risk rates to report to considering or expecting

parents.

Luckily, we are no longer in a position where the exact formulation of these models needs to concern us to any great degree, as researchers were when fitting complex segregation models. The vast majority of the effort to uncover the genetic architecture of CL/P now involves data from genome-wide genotyping arrays and high-throughput sequencing technologies, allowing us to obtain effect size estimates for genetic variants rather than relying on qualitative descriptors such as "major" genes or "many" genes of "small" effect. Presently, the data do seem to support the contribution of several genes having an outsized effect (recall from above that Ludwig et al. reported a GCTA analysis that showed roughly 31%–with an admittedly large standard error–of the estimated variance for risk in CL/P was attributable to common variants, with 25.5% contributed by only 24 loci). It seems likely that the remaining loci are of relatively small effect individually, but the possibility remains much of them are population (or even family) specific with appreciable effect size. These questions will be answered as genetic dataset sample sizes continue to grow, but it is important to remember that the prevalence rate of 1/1000, will continue to pose challenges in this regard.

1.5.3 Linkage Analysis

Prior to the advent of low-cost SNP arrays, one of the preferred methods was linkage analysis studies. Briefly, these aimed to identify genetic markers (typically microsatellites–a form of short tandem repeats that are highly variable between individuals and lend themselves to being tracked through families) that are inherited in tandem with a given disease or trait with families. The presence of this co-inheritance implies that the marker is located near the locus responsible for trait variation.

One of the first documented genetic genetic links with CL/P was a linkage analysis study that identified the locus responsible for coding the gene *TGFA* (Ardinger et al., 1989). This has since been replicated multiple times and in multiple populations (Woong Sull, 2009; Marazita, 2009; Ebadifar, 2015). While the loci nominated by other linkage analyses have proved less robust owing to restricted sample size, ground was gained in the aughts, when researchers combined their data, allowing them to find several loci that have proved broadly

reproducible. (e.g. *IRF6*, *MSX1*, *TGFB3*, *RARA*, and *BCL3*). [Marazita et al., 2004, 2009] While genetic investigation into CL/P today generally proceeds via methods like GWAS, or through sequencing studies like whole genome sequencing or whole exome sequencing (WGS, WES), linkage analysis studies still appear in the literature, sometimes with novel findings. For example, in 2016, Mohamad Shah et al. performed linkage analysis using data from eight large Malaysian families, provided confirmatory evidence for several known cleft loci, while nominating several candidate loci.

1.5.4 GWAS

The primary feature of linkage-based methods is the requirement that the data be strictly family-based. This poses a particular challenge when attempting to study rare diseases. While CL/P is, as is frequently pointed out in the literature, the most common congenital disease, it is important to remember that congenital diseases are themselves rare. The difficulty this poses is that cases of CL/P often appear only once in a family (termed "singletons") and hence provide no information about inheritance patterns. Population-based designs, now widely used in practice, offer a way around this difficulty.

1.5.4.1 The GWAS Model

Conceptually, GWAS is straightforward: it is simply an association test between each genotyped variant and a given phenotype in a sample, generally of "nominally" unrelated individuals (that is, individuals not related to each other by greater than some degree, often taken to be third). In the case of a quantitative phenotype, this amounts to a linear regression of the phenotype onto the number of copies of an allele (0,1,or 2). In the case of a binary phenotype (e.g. an individual either presents with an orofacial cleft or does not), the linear model can be generalized with a "link function" such that the outcome is the log odds of the phenotype. These models are easily extended to accommodate an arbitrary number of covariates, the type of which will generally depend upon the phenotype/study design in question, but virtually always include a way to control for genetic ancestry, which is well understood to pose a risk of confounding termed "population stratification." Mathematically,

this can be formulated as follows:

$$\mathbf{Y} = \mathbf{X}\beta + \epsilon \quad \text{(Quantitative)}$$
$$\log\left(\frac{p}{1-p}\right) = \mathbf{X}\beta \qquad \text{(Binary)}$$

where $\mathbf{Y}$ represents a vector of n x 1 phenotypes, $\mathbf{X}$ represents an n x p matrix indicating the number of alleles for each individual at each SNP (0, 1, or 2), β is a p x 1 vector of effect sizes, and ϵ is an n x 1 vector of residuals assumed to be independently and identically normally distributed with mean 0 and variance σ^2. In the binary case, p represents the probability of an individual having the phenotype of interest (e.g., presenting with an orofacial cleft). There are various downstream analyses that can be performed with these statistics, which will not be detailed here, but for a recent review see Uffelmann et al. (2022).

Among the earliest and most influential GWAS occurred in 2007, when the Wellcome Trust Case Control Consortium performed an analysis of 14,000 individuals for seven common diseases, establishing many of the procedures and protocols that are still considered standard practice in GWAS today. The first GWAS for CL/P followed two years later in 2009 (Birnbaum et al., 2009a, Birnbaum et al., 2009b, and Grant et al., 2009). These studies provided confirmatory evidence for the association for the long-implicated *IRF6* gene as well as the 8q24 locus. Several further GWAS followed (e.g. Beaty 2010, Mangold 2010, Ludwig, 2012), with an especially large string of successes occurring in 2017 (Holzinger et al., 2017; Leslie et al., 2017; Ludwig et al., 2017; Moreno Uribe et al., 2017; Yu et al. 2017).

In recent years, GWAS has continued to uncover novel findings, with special attention being given to sub-phenotype differentiation and population diversity. Mukhopadhyay et al. (2022) used a sample of approximately 7000 individuals of African, Asian, European, and Central and South American ancestry to provide insight into the distinct genetic architectures that underlie various CL/P subtypes (seven in total). They reported five novel loci and nine replications and additionally noted a large degree of heterogeneity for within-loci SNPs. Similarly, Robinson et al. (2023) conducted a trio-based GWAS of approximately 1300 individuals of African, Asian, Latin American, and European ancestry to investigate cleft palate, with a distinction between cleft soft palate (CSP) and cleft hard palate (CHP). They reported a novel locus (9q33.3, including the gene *ANGPTL2*) as being significantly

associated with CHP as well as 19 additional loci showing suggestive evidence of association with hard, soft, or either ("any cleft palate", ACP), as well as 22 SNPs showing "nominal" ($p < 0.05$) evidence of replication.

At present, there are approximately a dozen replicated genes implicated in CL/P, with a couple dozen further nominated genes (note: "nominated" is not meant to be read here as "not yet replicated" rather than "suggestively associated"–many of these have demonstrated significant association) awaiting further investigation. The most robust findings include *IRF6*, *PAX7*, *FOXE1*, *MAFB*, *NOG*, *MAFB*, and the 8q24 locus.

1.5.5 Interactions

The understanding that a large amount of phenotypic variance in CL/P is attributable to genetic variance falls short of a complete picture. Additionally, CL/P poses a substantial burden on those affected, their families, and the healthcare systems of their societies. While the simple identification of implicated loci leaves little room for therapeutic intervention, the question of what may be done to reduce risk changes in the context of gene x environment interactions. That is, if the effects of relevant genetic loci are, to some extent, dependent on the environment, elucidation of these interaction effects may lend themselves to insight that is actionable.

The role of non-additive (that is, dominant, epistatic, or gene x environment) variance has been of theoretical interest to geneticists since the inception of the field. While there are reasons to believe the majority of genetic variance for complex traits is additive (Hill et al. (2008)), plenty of exceptions have been documented and it has been suggested that G x E effects may be especially relevant in the etiology of human disease (Hunter (2005)). When exploring gene-gene and gene-environment interactions, researchers face various statistical hurdles. However, they can draw upon a rich body of existing literature to guide their decision-making process, helping them identify relevant variables and determine the most appropriate ways to incorporate these factors into their statistical models.

1.5.5.1 Gene x Gene

The current evidence for G x G effects (commonly termed "epistasis") in orofacial clefts is fairly limited. As was alluded to above, this does not necessarily imply their unimportance or absence, but rather due to the relatively small sample sizes in CL/P studies to begin with (owing to the condition's rarity) as well as the statistical challenges involved with robust detection of gene-gene interactions generally. A few studies have attempted to highlight potential gene-gene interactions, providing some suggestion for what we may expect to see in future studies.

Velázquez-Aragón et al. (2016) examined a set of 24 SNPs in 17 documented CL/P loci using 133 cases and 263 controls from a Mexican Mestizo population. Their approach involved the use of multifactorial dimensionality reduction and identified 6 significant pairwise interactions. The authors concluded by proposing a potential interaction network (centered around *IRF6*) that involved these interactions, noting that confirmatory evidence would still be needed. Zhou et al. (2019) focused specifically on gene-gene interactions among *SPRY* genes (*SPRY1*, *SPRY2*, and *SPRY4*—*SPRY3* was excluded as it is on the X chromosome) in a sample of 825 Europeans and 1038 Asians. After performing conditional logistic regression and correction for multiple testing, they found 14 significant interactions (10 in Europeans and 6 in Asians). Most recently, Chen et al. (2024) investigated interactions among 6 DNA repair genes in a sample of 806 Chinese trios. After correction, they also found 14 significant interactions, with a dozen of them involving *ATR* with either *ERCC4* or *XRCC2*. Like the previous two studies, the authors emphasized that the results would need follow-up and replication in order to consider the findings robust.

1.5.5.2 Gene x Environment

The current evidence for G x E effects implicated in orofacial clefts is similarly limited but growing. Wu et al. (2014) investigated the possible interaction between various loci and environmental tobacco smoke (ETS). They documented suggestive evidence for an interaction effect with ETS and the locus 4p16.1 in Asian trios with CP, highlighting markers in *SLC2A9* and *WDR1*. (Note: CP is generally considered distinct from CL/P, but more on this

in Phenotypes and Sub-Phenotypes section below). Haaland et al. (2019) proposed a method to identify parent-of-origin (PoO) interactions with environmental exposures (PoOxE). In a sample of 1908 European families, they reported suggestive evidence for a PoOxSmoke effect for the gene *ANK3* and a PoOxAlcohol effect for the gene *ARHGEF10*. Zhang et al. (2021) applied the genotypic transmission disequilibrium test to a sample of trios, with either syndromic or non-syndromic orofacial clefts. They reported suggestive evidence for two G x Smoking effects (*FGF12* and MUSK–interestingly, both protective), two G x Vitamin effects (*CASP9* and *RETREG1*), and one G x Alcohol effect (SLOCO3A1). In 2022, Carlson et al. performed a genome-wide interaction study on a European population with CL/P, following a 2-stage procedure. They first used a joint test to assess genetic (G) effects and gene x environment effects (GxE) across the entire genome to identify when genetic effects were either similar across all environments or specific to a particular environment. 127 loci that showed suggestive effects ($p < 0.05$) and these were then interrogated for GxE alone. Through simulations, they demonstrated that, conditional on significance at the first threshold, p-values of less than 2.74e-3 resulted in a false-positive rate of 0.05. Adjusting the simulation-based significance threshold for 572 tests yielded 4.8e-06, a threshold at which 40 loci were determined to be significant. Among these they highlighted interaction effects at locus 6q22 (near *VGLL2*) with G x Alcohol effects and 6p22.3 (near *PRL*) with G x smoking effects as particularly biologically plausible, citing experimental research from animal models.

1.5.6 Sequencing Studies and Functional Analysis

While the study designs highlighted above are undoubtedly a necessary first step towards a full understanding of the etiology of CL/P, they are largely mute on a central question–that of biological function. In segregation analysis, the mode of inheritance is the main object of discovery. In linkage analysis and more fine-grained association analysis (e.g. GWAS), identifying the specific loci or genetic variants responsible for CL/P risk is the main object of study. However, for the purposes of understanding what exactly is taking place at the molecular/biochemical level, different types of analyses are needed. In recent years there has

been much progress in this area, with research following up and building upon the discoveries of association studies involving omics data, gene/pathway analysis, and functional studies with model organisms such as mice and zebrafish.

An example of the power of these methods is Leslie et al. (2012), which sought to reconcile previous evidence for a role of *ABCA4* with the findings that this gene's expression was largely restricted to the retina (Beaty et al., 2010) and so unlikely to have a causal role in the development of orofacial clefting. The authors hypothesized that the signal detected in association studies from *ABCA4* was actually coming from one of the neighboring genes. *ABCA4* is flanked by the genes *GCLM* and *ARHGAP29*. Given that *GCLM* had previously shown no expression in craniofacial tissue (Diez-Roux et al., 2011) and the fact that the *GCLM* mouse did not appear phenotypically different than the wild-type (Yang et al., 2002), the authors proposed that instead *ARHGAP29*, a gene believed to be involved in various craniofacial development processes and located less than 50 kb away from *ABCA4*, was a more likely candidate. To investigate this hypothesis, the authors performed in situ hybridization and immunodetection on embryonic mouse tissues at various developmental stages and used a sample of case/control data to conduct two-phase mutation screening of *ARHGAP29* exons and used Fisher's exact test to compare their rare variant frequencies. The combined evidence of these demonstrated strong support for the causal role of *ARHGAP29* and implied the evidence pointing to *ABCA4* was likely an artifact of confounding by LD structure. This conclusion has since been confirmed by many studies (Letra et al., 2014, Savastano et al., 2016, Liu et al., 2017, Paul et al., 2017, Yu et al., 2020, Tang et al., 2020).

1.5.7 Animal Models

With low-cost SNP arrays and the availability of open-source statistical software for conducting association analysis, gene nomination/discovery has outpaced in vivo validation by many orders of magnitude. This asymmetry comes at a cost. While data on statistical association can sometimes be put to clinical use (e.g., with polygenic risk scores), the insights afforded by the validation of gene action in animal models offers information that is simply

unavailable through association. Here we will outline some of the key areas of animal research in the context of CL/P research: model organisms typically used, their advantages and disadvantages, and a look at what developments may be in store.

The common house mouse, *mus musculus* has a long history in genetics research and biology more broadly. As gene conservation tends to show a general (although not perfect) correlation with phylogenetic distance, it is no surprise that biologists would be interested in a model organism that belongs to the same class (Mammalia). Arguably the premiere mammalian model organism, it has been used for decades in diverse areas such as behavioral screens, toxicity assessment, and drug development. In 2000, Gong et al. first proposed the Twirler mouse as a model for the study of cleft lip and palate. Twirler (*Tw*) is a semidominant mutation that plays a key role in the formation of the mouse's midfacial region. Since then, several additional mutant strains have been introduced that exhibit cleft lip and palate phenotypes that resemble those seen in humans. Some of the implicated signaling pathways from these mice include *BMP*, *WNT*, and *p63/IRF6* (Juriloff & Harris, 2008).

An another animal model that has appeared frequently in the literature is *Danio rerio* (Zebrafish). In contrast to mouse models, Zebrafish reproduce more rapidly and have transparent flesh, allowing researchers to better observe the inner workings of their development. There are several reviews in the literature that highlight advances in orofacial genetics that have been provided by Zebrafish models as well as the drawbacks researchers should remain cognizant of (e.g. Li et al., 2021; Lieschke & Currie, 2007; Machado & Eames, 2017; and Raterman et al., 2021).

A less commonly used, but still important animal model is *Gallus gallus*, the chicken. Van Otterloo et al. (2016) detail some of the advantages offered by the chicken (e.g. easy accessibility for tissue manipulation and pathway conservation with mammals as well as some of their respective drawbacks (e.g. limited options with respect to transgenics and no fusion of the secondary palate). Wolf et al. (2014 & 2015) additionally identified the Nova Scotia Duck Tolling Retriever (NSDTR) as a potential animal exhibiting spontaneous alveolar clefts, although a recent Master's thesis by Yena Jun (Jun, 2020) highlighted significant differences between the NSDTR model and humans, specifically in terms of defect size and shape,

suggesting limited feasibility in attempts to use NSDTR in cleft reconstruction.

While research continues to explore possibilities for translational work on model organisms, investigators are well-advised to be mindful of the limitations. Among these, Cox (2015) highlights that many animal studies lack the desired level of precision in phenotyping animal models and the fact that that many published animal studies focus exclusively on homozygous null phenotypes, which pose problems generalizing to humans (typically heterozygous) and McGonigle & Ruggeri (2014) highlight the need for pharacokinetic/pharmacodynamic relationships when investigating new chemical entities for efficacy and toxicity.

1.5.8 Phenotypes and Sub-Phenotypes

Orofacial clefts are notably heterogeneous, with varying clinical presentations and underlying genetic architectures. Understanding the different phenotypes and sub-phenotypes of orofacial clefts is crucial for improving diagnosis, treatment, and research. Robinson, Curtis, and Leslie (2024) provide a comprehensive review of the current knowledge and open questions regarding the various subtypes of CL/P (CL, CP, and CLP).

The authors describe the embryological development of the lip and palate, which occurs in two main phases. The first phase, from weeks 4-6, involves the formation of the upper lip and the five facial primordia: the medial nasal processes, the lateral nasal processes, and the maxillary process. The second phase, from weeks 7-14, involves the development and fusion of the secondary palate. Traditionally, a distinction has been made between CL/P and CP based on differences in developmental timing, sex bias, co-occurrence of additional clinical features, and observed recurrence risks. The female delay in palatal fusion by approximately one week is thought to contribute to the higher prevalence of CP in females compared to CL/P.

When discussing subtyping schemas, Robinson et al. (2024) note that genetic overlap (shared risk loci between subtypes) is more commonly applied to CL and CL/P, while genetic heterogeneity (subtype-specific risk loci) is more often used to differentiate CL/P and CP. However, they propose a third model that may better align with current evidence. This model takes into account the evidence that contradicts the genetic overlap model for CP and CL/P,

such as the shared genetic risk factors *FOXE1*, 8q24.21 in a Han Chinese population, and *GADD45G* and *ARHGAP29* showing associations with both subtypes in separate studies. The authors also highlight the role of *IRF6* in "mixed clefting," where multiple subtypes occur within a single family.

In discussing cleft laterality, (that is, on what side of the cleft and/or palate the clefting phenotype is present), the authors raise the interesting point that it is likely non-random. In particular, clefting phenotypes are more commonly found on only one side than both and clefting phenotypes on the left side occur twice as frequently as those on the right. A recent review on sideness in unilateral clefting (Fell et al. 2023), failed to draw many definitive conclusions, but noted that increasing attention is being paid to the topic.

The authors conclude with a discussion of what they term the "subclinical OFC phenotype hypothesis," which proposes that some of the incomplete penetrance observed in multiplex families with OFCs could be explained by genetic carriers having a subclinical phenotype but being classified as unaffected. They emphasize the importance of engaging in defining and exploring associations with detailed phenotypes to better understand the complex genetic architectures underlying orofacial clefts. Their work provides a solid foundation for future research and clinical applications in this field.

1.5.9 Mendelian Randomization

While most studies on CL/P have focused on observational data, owing to the nature of Mendelian segregation, researchers are sometimes placed in a position to interrogate causal hypotheses. The most common way this is done in genetic epidemiology is through a method called Mendelian Randomization (MR). Borrowing the technique of instrumental variables (IVs) from econometrics, MR leverages the fact that Mendelian segregation can, in principle, behave as a "randomizing" mechanism, breaking the causal chain between anything causally upstream of the IV and a given "exposure".

1.5.9.1 MR Assumptions

There are many methods available for researchers to conduct MR analyses, but they all rely on a common set of assumptions. The first three are sufficient to test the null hypothesis of no association between the exposure and the outcome, an additional assumption, which may be called the point-estimate-identifying condition (Sanderson et al. 2022) is needed to ensure the validity of effect size estimates.

1. The IV must be associated with the exposure. This is often referred to as the "Relevance" assumption
2. There are no unmeasured confounders between the IV and the outcome. This is often referred to as this "Independence" or "Exchangeability" assumption.
3. The IV only affects the outcome through the exposure. This is often referred to as the "Exclusion" assumption.
4. Point-estimate-identifying condition
    - Homogeneity of effect
        - Form 1: The "effect" is the same for everyone (Interpretation: "causal effect")
        - Form 2: The "effect" does not depend on the value of the instrument (Interpretation: "population average causal effect")
    - Monotonicity in association between IV and the exposure.

The latter of these assumptions (that is, monotonicity) makes the assumption that the direction of the effect is the same for everyone and comes with the interpretation that the effect size is estimating the "causal effect on those in the population who are affected".

1.5.9.2 MR Studies Involving CL/P

Rare for the MR literature, to date only two MR studies on CL/P have been conducted. The first was conducted in 2020 by Dardani et al. and had the impetus that there had been previous findings suggesting individuals with CL/P had on average, lower educational attainment (EA) and intelligence quotients (IQ) than unaffected individuals. The study had a sample size of 5951 (1692 cases and 4259 controls) and consisted of a GWAS meta-analysis,

a genetic correlation analysis carried out with linkage score disequilibrium regression, and finally an MR analysis to assess the causal effect of associated variants on EA. The conclusion from the study was that confidence intervals for genetic correlation estimates and MR all contained 0 and that the variants nominated by the GWAS were unlikely to place a causal role with EA or IQ.

The second study in the literature is a Master's Thesis completed by Anne Havlick in 2023 (Havlick 2023). Citing previous findings that individuals with CL/P have twice the prevalence of depression compared to unaffected individuals, she sought to assess the validity of these through an inverse-variance weighted two-sample MR analysis. (Note "two-sample" here indicates that the samples in which associated variants for the exposure and outcome are different). Among the study's merits were the use of several genetic ancestry groups (specifically European, East Asian, and African), sensitivity analyses to investigate the threat to validity posed by horizontal pleiotropy, and a frank admission of difficulties to interpretation imposed by varying phenotypic definitions among the groups (European individuals were phenotype via self-report, East Asian individuals by a combination of health records and self-report, and African individuals by a single-item questionnaire). The estimates reported in the study were null for the European and East Asian groups, while effect size African group was reported as 1.28 (0.94-1.75), albeit with a potential bias due to horizontal pleiotropy implied by one of the sensitivity analyses (specifically, that assessed via MR-Egger). Havlick concluded with a call for further investigation into the causal relationship between CL/P and depression when data for more robust inference is available.

1.6 Conclusion

This literature review has sought to provide a comprehensive overview of the current state of knowledge regarding cleft lip with or without cleft palate (CL/P). We have examined the epidemiology, psychology, healthcare costs, genetics, and various research approaches used to study this common congenital anomaly.

The epidemiological data shows that CL/P affects approximately 1 in 1000 live births

globally, with some variation across populations. Recent studies have shed light on risk factors such as maternal age, obesity, and smoking, though the exact mechanisms remain unclear. Psychologically, while some studies suggest increased risk for emotional and behavioral problems in children with CL/P, the evidence is mixed and effect sizes are generally small. More high-quality research is needed to clarify the psychological impacts. The substantial healthcare costs associated with CL/P treatment highlight the economic burden on families and healthcare systems, emphasizing the need for continued efforts to improve access to care and treatment options. Genetic studies have made significant strides in understanding the complex etiology of CL/P.

Heritability estimates are high, and genome-wide association studies have identified several robust genetic loci associated with increased risk. However, much of the genetic architecture remains unexplained, suggesting a role for rare variants and gene-environment interactions. Animal models, particularly in mice and zebrafish, have provided valuable insights into the developmental processes underlying CL/P. These models continue to be refined and offer promise for translational research. The field has begun to recognize the importance of phenotypic heterogeneity in CL/P, with efforts to better define and study various subtypes. This approach may lead to more targeted interventions and improved understanding of the underlying biology. While Mendelian Randomization studies on CL/P are limited, they represent a promising approach for investigating causal relationships between CL/P and various outcomes.

In conclusion, while significant progress has been made in understanding CL/P, many questions remain. Future research should focus on integrating findings from diverse approaches, including genomics, animal models, and epidemiology, to develop a more comprehensive understanding of CL/P etiology. This knowledge will be crucial for improving prevention, diagnosis, and treatment strategies, ultimately enhancing the lives of individuals affected by this condition.

2.0 Genome-wide Association Study and Downstream Analyses in Filipino Sample

2.1 Précis

This genome-wide association study (GWAS) investigated the genetic architecture of cleft lip with or without cleft palate (CL/P) in a Filipino cohort of 1,399 unrelated individuals (882 cases, 517 controls). The study identified a novel locus, *KCNQ5*, and replicated associations at three known loci: *ARHGAP29*, *IRF6*, and *SHROOM3*. A gene-based test using mBAT-combo highlighted potential masking effects in several genes, including *IRF6*. A transcriptome-wide association study (TWAS) further supported the involvement of known clefting loci *VAX1*, *NTN1*, and *IRF6*. Fine-mapping analyses and conditional and joint multiple-SNP analysis (COJO) provided additional insights into the putative causal variants at the associated loci, particularly highlighting the potential role of rs2235371, a missense variant in *IRF6*, based on its strong functional evidence and high linkage disequilibrium with the most significant signal. These findings contribute to the growing understanding of the genetic basis of CL/P, underscore the importance of conducting genetic studies in diverse populations, and provide a foundation for future research aimed at elucidating the etiology of this condition and improving strategies for prevention, diagnosis, and treatment.

2.2 Introduction

Cleft lip with or without cleft palate (CL/P) is one of the most prevalent congenital anomalies globally, resulting from the failure of lateral and medial nasal processes to merge properly in early embryological development. Incidence of CL/P varies among ancestry groups, with individuals of Asian ancestry commonly characterized as one of the groups greatest at risk (Tanaka et al., 2012). In the Philippines, it is estimated to occur in approximately 1 in 500 live births (Murray et al., 1997). Affected individuals experience health

and psychosocial consequences, creating an imperative for further research into its etiological origins and strategies for predicting risk.

Non-syndromic CL/P accounts for the majority of cases and is distinguished by the absence of other manifestations. Evidence supports the roles of environmental, genetic, and interaction (genetic x environment) factors in CL/P risk, though the exact interplay remains an area of active research.

Family-based methods have estimated higher concordance among monozygotic than dizygotic twins suggesting a significant heritable component to CL/P variation. Moreover, several dozen associated loci have been identified, to date (Ludwig et al., 2017). However, cumulatively, the estimated phenotypic variance explained by these associated loci remains low ($\approx 25 - 30\%$), while estimated heritability is $\geq 90\%$ (Grosen et al., 2011).

Elucidating the genetic architecture of CL/P presents multiple challenges, including limited sample sizes, diverse phenotypic presentations, and ambiguities surrounding the precise relationships among subphenotypes. A promising strategy to advance our understanding is to focus on populations with documented higher incidence rates, like those in East Asia. A further justification for collection and analyzing genetic data from this geographical region is the now well-appreciated problem in the human genetics community of the overrepresentation of individuals of European ancestry (Martin et al., 2017). Indeed, this was one of the principal reasons for the organization and maintenance of the "GWAS Diversity Monitor" (`https://gwasdiversitymonitor.com/`). Currently, The GWAS Diversity monitor records representation in GWAS by "supergroups" (i.e. European, Asian, African, African-American or Afro-Caribbean, Hispanic or Latino, and Other/Mixed). Despite making up over half of the global population, the total representation of individuals with Asian ancestry amounts to only 3.7% of total GWAS participants. While recent years have shown progress in this respect with the establishment of several large biobanks in Asia, individuals with specifically Filipino ancestry remain less represented still. Therefore, we investigated the genetic architecture CL/P in recruited participants from the Philippines through the analysis of a case-control GWAS.

2.3 Methods

2.3.1 Genotyping and Quality Control

Genotyping was performed by the Center for Inherited Disease Research (CIDR) on a total of 4,114 multiethnic samples using the Infinium Global Diversity Array-8v1.0, which targets clinical variants and is designed to capture genetic variation from diverse ancestries, followed by imputation using the TopMed Reference Panel (version 2). Imputed genotypes were then filtered based on a genotype probability threshold of 90%, followed by quality checks performed in collaboration with CIDR, including standard checks for call rate, error rate, sex check, relatedness, population structure, batch effects, Mendelian errors, and deviation from Hardy-Weinberg equilibrium.

2.3.2 Population Structure Analysis

To assess population structure and identify potential outliers, principal component analysis (PCA) was performed on the Philippines case-control sample. Initially, a set of 77,052 independent SNPs was selected by filtering for minor allele frequency (MAF ≥ 0.01) and pairwise LD ($R^2 \leq 0.1$) using PLINK software.

PCA was first conducted on the Philippines sample alone (2,174 subjects), using kinship estimation to categorize subjects as unrelated or related. This analysis identified five outlier individuals. Parallel coordinate plots and biplots of the first seven principal components (PCs) were inspected, stratifying by phenotype (CL, CP, CLP, unaffected) and DNA source (blood, blood spot, buccal cells, saliva) to assess systematic allele frequency differences, but none were detected.

Subsequently, PCA was performed on the combined dataset of the Philippines sample and 1000 Genomes Project data (3,202 subjects), with kinship estimates recomputed to classify subjects as unrelated or related. Three of the five previously identified outliers were found to map to populations other than the Philippines, while the remaining two were undetected in this analysis.

For the genome-wide association study (GWAS), 1,464 cases and controls were selected,

excluding the five outlier subjects. PCA was rerun using the related/unrelated groupings determined in the previous step. One additional outlier was identified, resulting in a final GWAS sample of 1,463 cases and controls. Of these, 517 were controls and 882 where phenotyped as CL/P yielding a total sample size of 1,399.

A scree plot of the PCs from the final GWAS sample was examined to determine the number of PCs required to adequately account for population admixture. Based on this assessment, five PCs were deemed sufficient for inclusion as covariates in the association analysis.

2.3.3 Statistical Analyses

2.3.3.1 GWAS

The statistical analysis of the case-control study was performed using the fastGWA-GLMM method implemented in the GCTA software package (Yang et al., 2011). Briefly, this analysis scheme utilizes a sparse genomic relationship matrix (GRM) computed from the genotype data to capture pedigree relatedness among individuals. It first estimates the variance components and other model parameters under the null generalized linear mixed model (GLMM) without including the genotype being tested. It then performs score tests for each variant based on the parameter estimates from the null model (Jiang et al., 2021). This offers a computational advantage as the null model parameters only have to be estimated a single time, rather than for every SNP tested. FastGWA-GLMM also applies saddlepoint approximation (SPA) to calibrate p-values and control false positive rates for binary traits with unbalanced case-control ratios (Jiang et al., 2019). Previous studies have shown that fastGWA-GLMM is computationally efficient compared to other GLMM-based methods and produces well-calibrated test statistics for both common and rare variants in biobank-scale datasets with some degree of relatedness (Gurinovich et al., 2022).

The selection of variables to include in a model as covariates should be based on principled statistical arguments. It has been shown that the inclusion of covariates can reduce power in case-control GWAS, especially in the setting of ascertained data on rare (prevalence $< 20\%$) diseases (Pirinen et al., 2012; Melford and Witte, 2012). As this description matches our

study design and the prevalence of CL/P in individuals of Filipino ancestry (estimated as 0.2% in people of Filipino ancestry), the decision was made not to include sex as a covariate. Additionally, as is shown in the supplementary materials of Pirinen et al. (2012), if genotype is independent of a given covariate, that covariate does not pose a threat of confounding the genotype-phenotype association. To evaluate this assumption, we conducted a GWAS using sex as the "phenotype," including only the principal components as covariates. The results were consistent with the null hypothesis, verifying that the exclusion of sex as a covariate was unlikely to bias estimates obtained from our GWAS (see Manhattan Plot in Appendix A).

2.3.3.2 TWAS

To further interrogate the genetic basis of the CL/P in our sample, we performed a transcriptome-wide association study (TWAS) using the S-PrediXcan software (Barbeira et al., 2018). S-PrediXcan uses pre-trained gene expression prediction models (e.g. from GTEx) and GWAS summary statistics to infer gene-trait associations.

Caution when interpreting the results of TWAS is warranted. As shown by de Leeuw et al. (2023), the traditional TWAS framework does not actually test the relationship between gene expression and the phenotype, despite sometimes being interpreted that way. Rather, what it tests is the relationship between *predicted* gene expression and the phenotype. As the gene expression is predicted out-of-sample, it necessarily includes some degree of measurement error, owing to the differences in gene expression between the sample used to estimate expression levels and the sample used to predict expression levels.

2.3.3.3 Gene-based Test

Gene-based tests were performed using mBAT-combo, a recently developed method that aggregates multiple SNP-trait associations into gene sets and is well-powered to detect associations in the presence of "masking effects" (Li et al., 2023). Masking effects occur when a gene contains multiple causal variants whose effects are obscured by linkage disequilibrium (LD) between them.

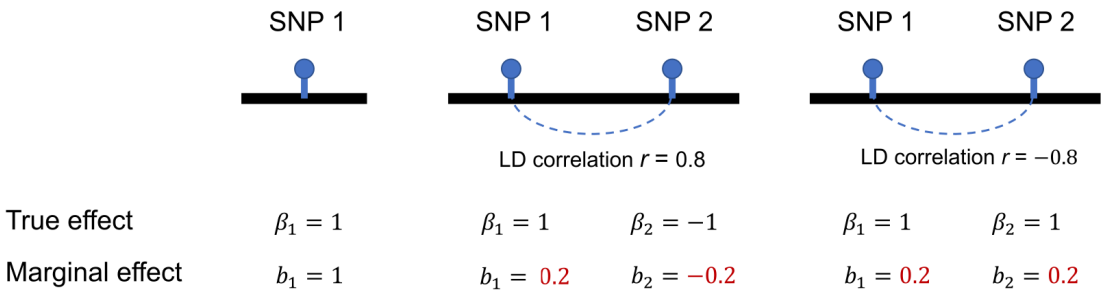

Figure 3: The masking effect occurs when the product of the true effects and the LD correlation is negative. The marginal effect of each SNP can be written as the sum of true effects weighted by the LD correlation between the two SNPs: $b_i = \beta_i + r \times \beta_j$. The marginal effect size is smaller than the true effect size when the SNP is masked by another SNP in LD with it (Jiang et al., 2023).

Most gene-based tests (e.g. MAGMA, VEGAS, GATES), assess gene-phenotype association by aggregating χ^2 statistics across SNPs included in a gene. While this strategy can provide improved power when multiple causal SNPs are contained within a gene, it loses power when the product of the SNP effects and the correlation between them is negative. For example, if two causal SNPs have opposite effect directions but positive LD, or same effect direction but negative LD, their marginal effects estimated from single-SNP models will be diminished and thus harder to detect. By using a multivariate approach that models the LD structure while accounting for effect directions, mBAT can capture masked effects that get canceled out in the univariate sum-of-squares approach. Intuitively, mBAT infers the total variance explained by all SNPs in a gene set, including LD-dependent covariance terms ignored by the sum-of-squares approach. Masked effects contribute positively to this joint variance, allowing mBAT to detect associated gene sets that would otherwise be missed. In extensive simulations and applications to real data, mBAT-combo demonstrated superior power over other methods, suggesting pervasive masking effects that may explain some of the missing heritability of complex traits.

2.3.3.4 Fine-mapping

To identify putative causal variants underlying the GWAS associations, statistical-fine mapping was performed using the echolocatoR R package (Schilder et al., 2022). EcholocatoR integrates multiple fine-mapping tools, including Approximate Bayes Factors (ABF), FINEMAP (Benner et al., 2016), SuSiE (Wang et al., 2020), and PAINTOR (Kichaev et al., 2014; Kichaev and Pasaniuc, 2015; and Kichaev et al., 2017), into an automated end-to-end pipeline. It extracts locus-specific statistics from a GWAS summary statistics file, computes LD matrices from publicly available repositories (UKBB, 1000 Genomes), implements the fine-mapping methods specified, and outputs plots showing the results from the methods as panels.

The loci prioritized for fine-mapping were selected based on the GWAS results, which included the three significant associations, as well as the suggestively significant association which was supported by previous findings in the literature. For each locus, a 10Mb window centered on the lead SNP was used. The EAS subset of the 1000 Genomes Phase 3 reference panel was used to estimate LD and the fine-mapping approaches selected were FINEMAP and SuSiE.

2.3.3.5 COJO

Conditional and joint multiple-SNP analysis (COJO) (Yang et al., 2012) enables a dissection of local genetic signals prioritized by GWAS. Providing an approximation to multiple regression analysis by leveraging local LD, it can identify secondary association signals at a locus that may not be discernible through standard (marginal) GWAS alone. It yields estimates in the form of "conditional" and "joint" effect sizes. The conditional effect size represents the effect of a SNP on the trait, adjusting for the effects of other SNPs included in the model, quantifying the association between a SNP and the phenotype that is independent of the signals from other nearby variants. The joint effect size is the effect of a SNP on the trait, estimated simultaneously with the effects of other SNPs in the model, quantifying the unique contribution of each SNP while accounting for the correlation structure among them.

2.4 Results

2.4.0.1 GWAS

We conducted a genome-wide association study (GWAS) on 1,399 unrelated individuals (882 cases/517 controls) recruited from the Philippines. The top five principal components of ancestry were included as covariates. One novel association was identified: (*KCNQ5*, p = 2.97e-08). Significant associations were observed for two known cleft loci: *ARHGAP29* (p=3.94e-08) and *IRF6* (p=1.18e-08), while another was observed at a suggestive threshold: *SHROOM3* (p=2.11e-07). [See Figure 4]

ARHGAP29 was first observed to be associated with CL/P in Beaty et al., 2010. As outlined above, the implicated gene at the time was thought to be *ABCA4*, but this was subsequently corrected through a closer examination in Leslie et al., 2012, which additionally suggested a pathway in which *IRF6* was posited to interact with the Rho pathway via *ARHGAP29*. It has since been replicated in Yu et al. (2017). *IRF6* is one of the most robustly-confirmed clefting loci in the literature. First proposed in a linkage study, (Marazita (2009)), it has since been replicated several times (e.g. Birnbaum et al., 2009; Beaty et al., 2010; Mangold et al., 2010) and has been the focus of a number of follow-up studies, which have further solidified the role of *IRF6* in the pathogenesis of CL/P (see Zhang et al., 2024 for a recent, comprehensive review). Additionally, population-specific studies have provided insights into the role of *IRF6* variants in different ancestries including Han Chinese (Li et al., 2023); South African (Naicker et al., 2023); and Malaysian (Nasroen et al., 2023). *SHROOM3* was nominated as a potential associated locus in Leslie et al., 2017 and confirmatory evidence has come to light since then (e.g. Diaz et al., 2023), Deshwar, 2023).

KCNQ5 codes for a potassium voltage-gated channel (subfamily Q member 5). GWAS Catalog lists 136 associations spanning 95 studies that document association with SNPs in *KCNQ5*. Among the associated traits are BMI, height, lung function, and schizophrenia. Additionally, there are a variety of ocular disorders associated with this gene, with the majority of the most significantly associated SNPs relating to phenotypes associated with vision. It is unclear how this locus is implicated in CL/P and further investigation will be

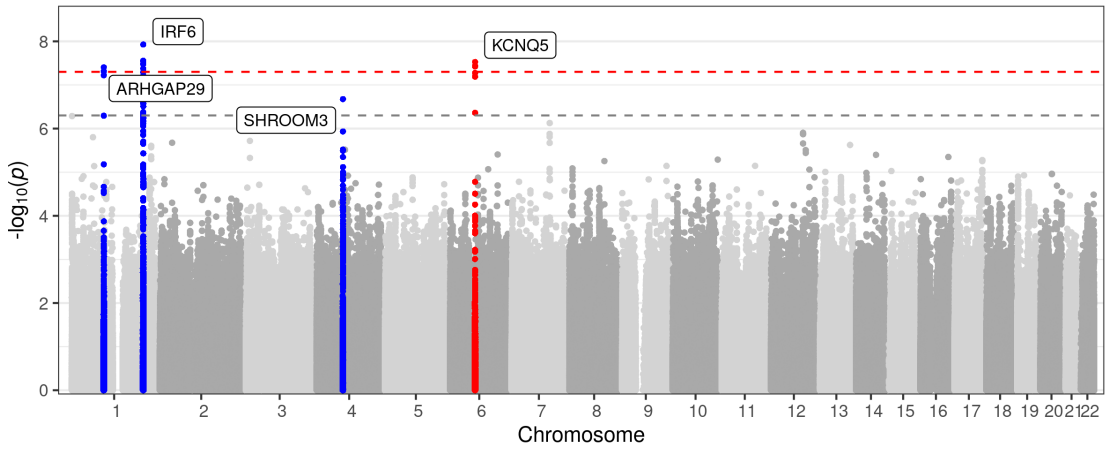

Figure 4: Manhattan Plot for Case-Control GWAS. Blue indicates known loci, red indicates novel. Dashed lines indicate significance thresholds: red indicated significant ($p = 5e-08$), grey indicates suggestive ($p = 5e-07$).

required to determine the validity of its associations and/or etiological relevance.

2.4.0.2 TWAS

To further interrogate the genetic architecture of CL/P in our sample, we performed a transcriptome-wide association study (TWAS). A significance threshold of 2.47e-06 was chosen to adjust for multiple testing of 20,215 genes. At this significance threshold, 21 genes were determined to be significant. Notably, among these were previously confirmed clefting loci *VAX1* ($p = 2.4e-06$, mean z-score = -3.05), *NTN1* ($p = 1.50e-10$, mean z-score = 0.70), and *IRF6* ($p = 1.2e-06$, mean z-score = 2.30), a known clefting locus determined to be significant in our GWAS. Additionally present were *SYT14* ($p = 1.12e-06$, mean z-score = 0.70) (a gene just downstream of *IRF6*) as well as *PLEKHN1* ($p = 7.22e-07$, mean z-score=0.70), which came quite close to the suggestive threshold for significance in the GWAS ($p = 5.17e-07$). Notably, these six genes were among the top seven significant loci when ranked by absolute mean z-score (Figure 6)

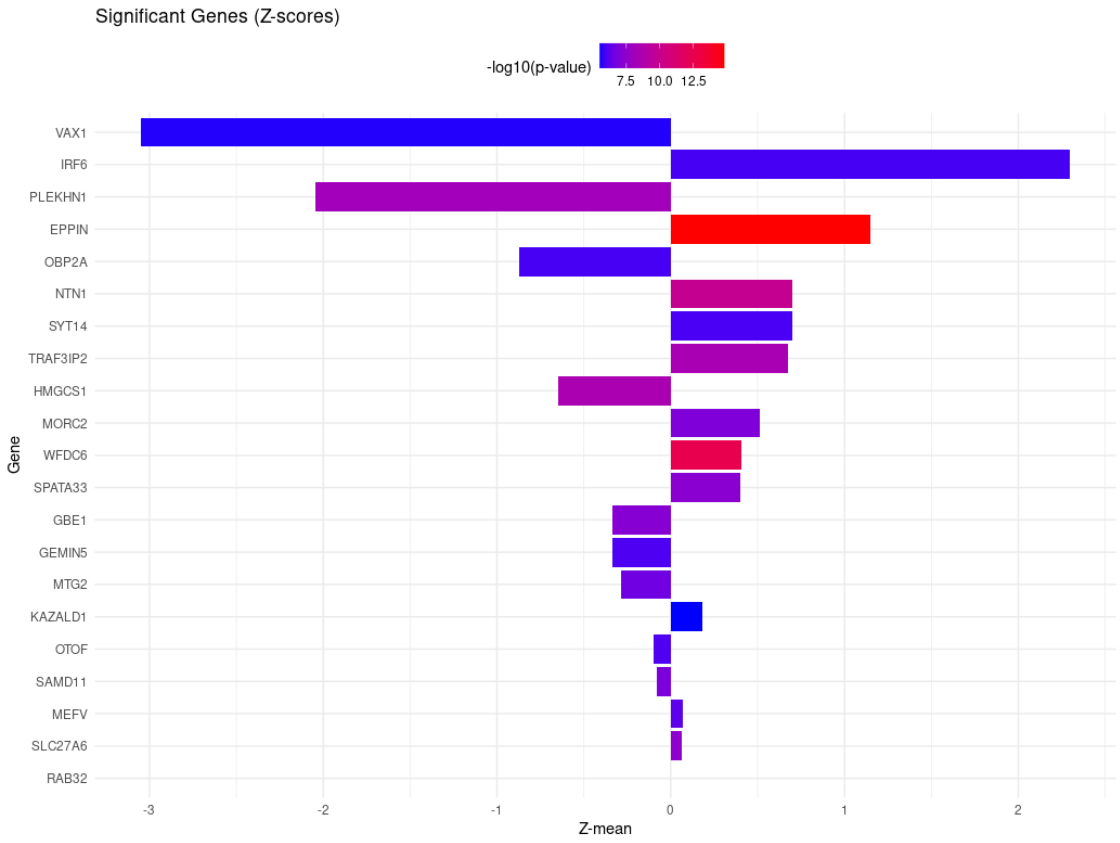

Figure 5: TWAS Results: significant genes ($p < 2.47e-06$) ranked by absolute mean z-score.

2.4.0.3 Gene-based Test

Following up the results of the GWAS, we conducted a set-based test to identify significant gene-level associations with CL/P. A significance threshold of 2.9e-06 was chosen to adjust for multiple-testing of 17,271 genes. At this threshold, two genes were significant: *C1orf74* (p = 9.27e-07) and *UTP25* (p = 5.88e-07) [Figure 5]. Additionally, to assess potential masking effects, the p-values calculated from mBAT (p-mBAT) and fastBAT (p-fastBAT) were assessed. As outlined above, in the presence of masking effects, p-mBAT had greater power than p-fastBAT. It follows from this that if a gene is determined to be significant by p-mBAT, but fails to reach significance by p-fastBAT, this is suggestive of the presence of masking effects. Four genes met this criterion: *C1orf74*, *TRAF3IP3*, *IRF6*, and *UTP25* (Table 1).

2.4.0.4 Fine-mapping

In light of the results from the gene-based test, implying potential masking effects contained in the region surrounding *IRF6*, we explored the results of this fine-mapping analysis in more detail. The results of the fine-mapping analyses of *ARHGAP29*, *SHROOM3*, and *KCNQ5* are contained in Appendix A.

The results for the fine-mapping analysis for the 1Kb region centered on the top SNP in the *IRF6* locus are shown in Figure 7. Initial analysis of the region revealed the interesting finding that a particular SNP: rs614662, ranked low in the initial GWAS, was assigned the highest posterior probability (PP) by FINEMAP and a PP of 1.0 by SuSiE, despite the presence of several SNPs with more significant p-values in the region (Figure 8).

However, further analysis revealed that this was likely an artifact of the fine-mapping process, wherein some SNPs are dropped from the dataset in order to create a "sparse" LD matrix. The LDlink tool, LDProxy (`https://ldlink.nih.gov/?tab=ldproxy`) was used to look for proxy SNPs (SNPs with high LD) for rs614662. 1000G was selected as the reference with the EAS subpopulation and a window of 2Kb was selected to filter for proxies. This search revealed that rs614662 was in complete LD (D'=1.0) with the SNP rs2235371, a missense variant with a Genomic Constraint score of (Z=4.83) of the surrounding 1Kb region,

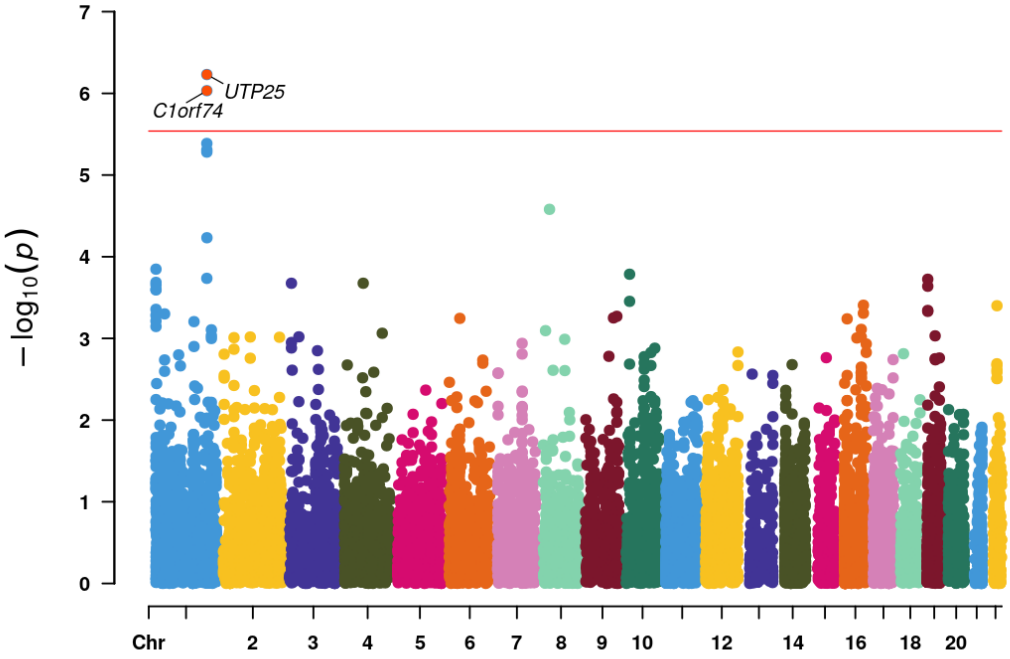

Figure 6: Manhattan Plot for Gene-based Test. Red line indicates significance threshold of 2.9e-06.

Table 1: Gene-based test: potential masking effects. (**bold** indicates significant values)

Gene	p-mBAT	p-fastBAT	p-mBATcombo
C1orf74	**4.65e-07**	1.08e-04	**9.27e-07**
TRAF3IP3	**2.07e-06**	1.99e-04	**4.10e-06**
IRF6	**2.53e-06**	8.40e-05	**4.92e-06**

corresponding to the top 1% of non-coding regions in the genome. A 2x2 allele frequency table from the LDPair tool (also part of LDlink) shows that out of 1008 samples, there are 0 T/T genotypes at these particular SNPs. Additionally, gnomAD (`https://gnomad.broadinstitute.org/`) reports a CADD Combined Annotation Dependent Deletion) score of 23.5, suggesting this variant is predicted to be among the top 0.5% most deleterious substitutions in the human genome, a REVEL (Rare Exome Variant Ensemble Learner) score of 0.385, indicating a moderate likelihood of the variant being pathogenic, a phyloP score of 8.90, indicating this region of the genome is highly conserved across species (suggesting strong evolutionary constraint), and a PolyPhen (max) score of 0.758, suggesting this variant is possibly damaging to protein function. Further, both gnomAD and and 1KG show the East Asian ancestry group as having a much greater allele frequency for this SNP, followed by the European ancestry group, and being least frequent in the African ancestry group, interestingly paralleling the prevalence rates reported in the literature.

2.4.0.5 COJO

Following up on the results of the fine-mapping analysis outlined above, COJO analysis was first carried out using the "condition on a single SNP". A 500Kb region centered on rs614662 was extracted and independent analyses were run conditioning on rs614662 and rs2235371. Consistent with the above discussion, when conditioning on rs614662, 27 SNPs were significant ($p < 5e-08$), while when conditioning on rs2235371, no SNPs were significant ($p_{\min} = 0.522$). As the most significant SNP in the rs614662-conditioned model

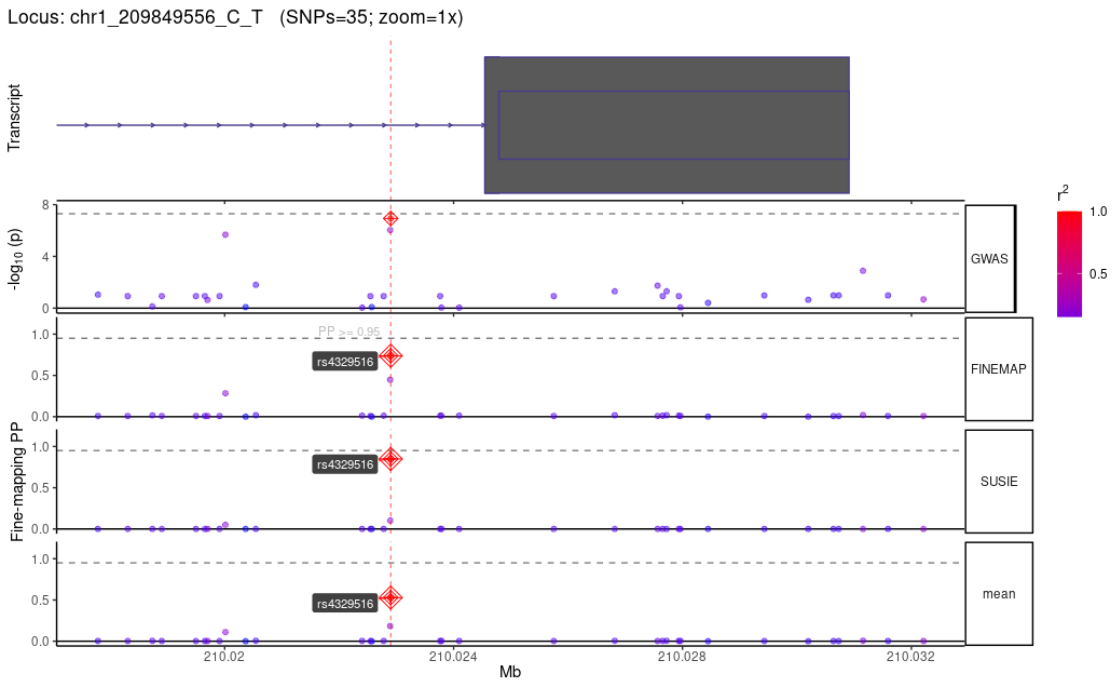

Figure 7: Results of the fine-mapping analyses for the 1Kb region centered on the most significant SNP in *IRF6*.

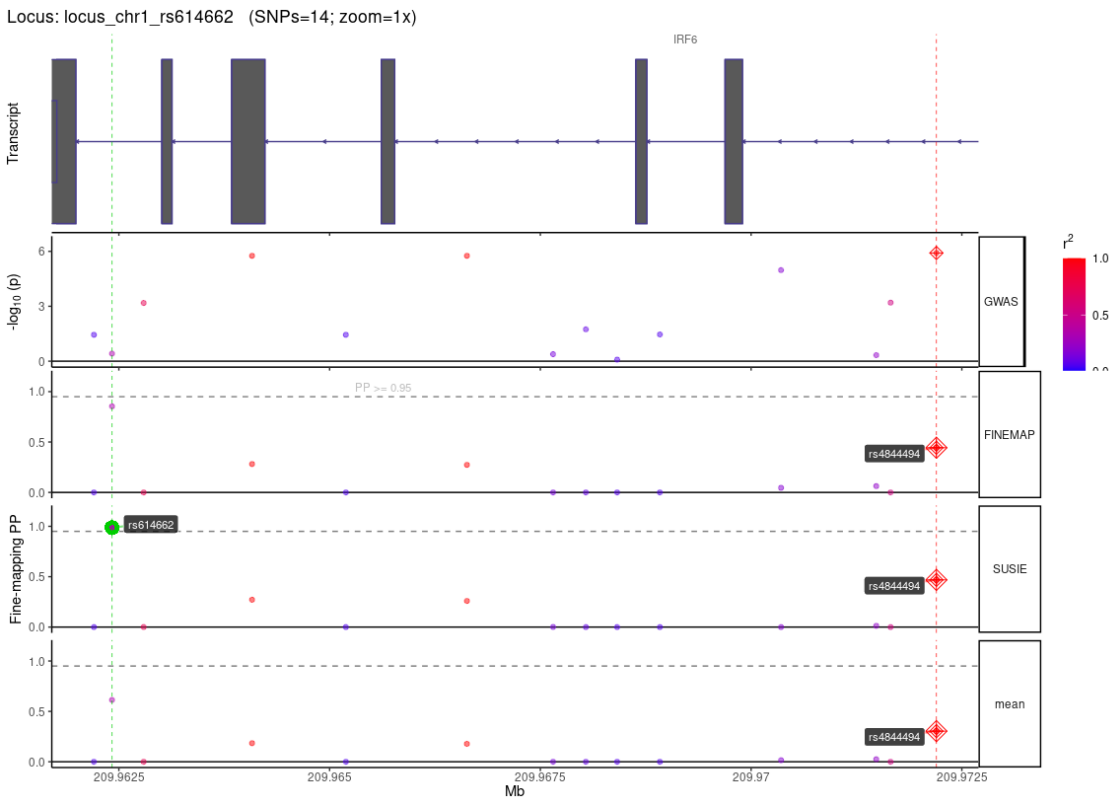

Figure 8: rs614662, ranked extremely low in the initial GWAS, determined by FINEMAP to be the SNP with the greatest posterior probability (PP) in the region, while SuSiE determined its PP to be 1.0.

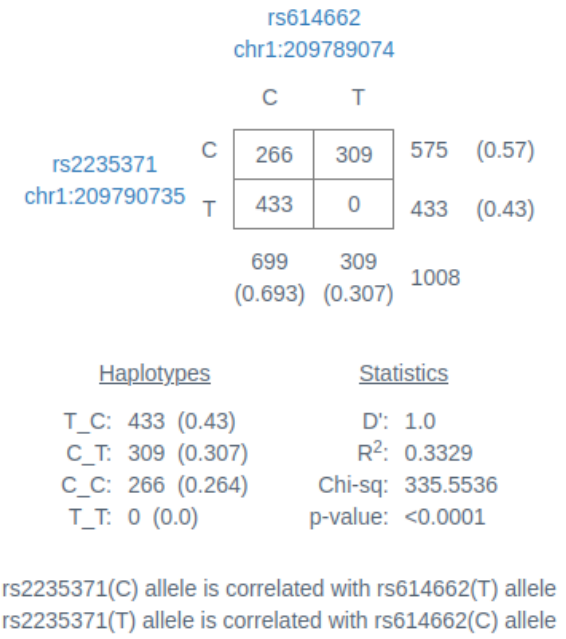

Figure 9: Allele frequency table from LDPair.

was rs4329516, a further single SNP model was run condition on rs4329516. As expected, the results yielded no significant SNPs.

As the above COJO results imply the significance of at least one of either rs2235371 or rs4329516, a 1Mb region centered on the midpoint between the two SNPs was then extracted and analyzed. Given the high LD between the two SNPs, the COJO parameters were set to a window of 10000Kb (10 Mb), assuming that SNPs more 10 Mb apart are in complete linkage equilibrium and a lowered the collinearity threshold from the default 0.9 to 0.7, allowing more SNPs to enter the model simultaneously. The results from the joint analysis revealed that only rs4329516 was significant ($p = 1.18e - 08$), with no significant results from the conditional analysis.

While this analysis implies the relevance of rs4329516, its functional evidence is far less persuasive than rs2235371.

2.4.1 Discussion

GWAS of CL/P in a Filipino sample identified a novel locus *KCNQ5*, while also replicating three known loci, *ARHGAP29*, *IRF6*, and *SHROOM3*. The gene-based test using mBAT-combo further highlighted potential masking effects in *C1orf74*, *TRAF3IP3*, *IRF6*, and *UTP25*. The transcriptome-wide association study (TWAS) also identified several significant genes, including previously confirmed clefting loci *VAX1*, *NTN1*, and *IRF6*.

The identification of novel locus *KCNQ5* warrants further investigation into their potential roles in the etiology of CL/P. While *KCNQ5* has been associated with various traits such as BMI, height, lung function, schizophrenia, and ocular disorders, its specific involvement in craniofacial development remains unclear.

The replication of known loci *ARHGAP29*, *IRF6*, and *SHROOM3* in this Filipino sample supports the idea that there are shared genetic risk factors for CL/P across different populations. However, the identification of novel loci also suggests the presence of population-specific risk variants, highlighting the importance of conducting genetic studies in diverse populations to better understand the genetic architecture of complex traits like CL/P.

The gene-based test using mBAT-combo identified potential masking effects in several genes, including the known clefting locus *IRF6*. These findings suggest that the presence of multiple causal variants within a gene, with opposing effect directions or LD patterns, may obscure the true associations when using traditional single-SNP approaches. Further investigation of these masking effects may uncover additional genetic risk factors for CL/P and contribute to explaining the missing heritability of this complex trait.

The TWAS results provide additional support for the involvement of several known clefting loci, such as *VAX1*, *NTN1*, and *IRF6*. However, it is important to interpret these results with caution, as TWAS tests the association between predicted gene expression and the phenotype, rather than directly testing the relationship between actual gene expression and the phenotype. The measurement error introduced by out-of-sample gene expression prediction should be considered when interpreting TWAS results.

Fine-mapping analyses, particularly for the *IRF6* locus, provided insights into the putative causal variants underlying the GWAS associations. The missense variant rs2235371

emerged as a strong candidate due to its complete linkage disequilibrium (LD) with the top signal (rs614662) and compelling functional evidence, including a high CADD score, REVEL score, and phyloP score, indicating its potential deleterious impact on protein function and evolutionary conservation. The allele frequency differences across ancestry groups for this variant also parallel the reported prevalence rates of CL/P, further supporting its potential role in the etiology of the condition.

The COJO analysis further investigated the *IRF6* locus, aiming to identify secondary association signals that may not be discernible through standard GWAS. The results from the joint analysis revealed that only rs4329516 was significant, while no significant results were observed in the conditional analysis. Although this analysis implies the relevance of rs4329516, its functional evidence is less persuasive compared to rs2235371. Further studies are needed to clarify the relative contributions of these variants to CL/P risk.

2.4.2 Conclusion

This GWAS of CL/P in a Filipino sample identified a novel risk locus, *KCNQ5*, replicated associations at three known loci (*ARHGAP29*, *IRF6*, and *SHROOM3*), and highlighted potential masking effects in several genes, including *IRF6*. The TWAS further supported the involvement of known clefting loci, while fine-mapping analyses and COJO provided insights into putative causal variants, particularly the missense variant rs2235371 in *IRF6*, which showed strong functional evidence and complete LD with the top signal. These findings demonstrate the importance of conducting genetic studies in diverse populations to better understand the genetic architecture of complex traits like CL/P and to address disparities in genetic research. The identification of both shared and population-specific risk variants in this Filipino sample underscores the need for more inclusive genetic studies to fully elucidate the genetic basis of CL/P. Future studies should focus on functionally characterizing the novel locus *KCNQ5*, investigating the potential masking effects identified by the gene-based test, and further exploring the relative contributions of rs2235371 and rs4329516 in *IRF6* to CL/P risk. Additionally, integrating genetic data with other omics data, such as transcriptomics and epigenomics, may provide a more comprehensive understanding of the biological

mechanisms underlying CL/P. In conclusion, this study contributes to the growing body of knowledge on the genetic basis of CL/P and highlights the importance of conducting genetic studies in diverse populations. The findings reported here provide a foundation for future research aimed at better understanding the etiology of this complex trait and ultimately developing improved strategies for prevention, diagnosis, and treatment.

Appendix A Supplementary Material

It has been shown that the inclusion of covariates can reduce power in case-control GWAS, especially in the setting of ascertained data on rare (prevalence $< 20\%$) diseases (Pirinen et al., 2012, Melford and Witte 2012). As this description matches our study design and the prevalence of CL/P in individuals of Filipino ancestry (estimated as 0.2% in people of Filipino ancestry), the decision was made not to include sex as a covariate. Additionally, as is shown in the supplementary materials of Pirinene et al., (2012), if genotype is independent of a given covariate, that covariate does not pose a threat of confounding the genotype-phenotype association. To evaluate this assumption, we conducted a GWAS using sex as the "phenotype," including only the principal components as covariates. The results were consistent with the null hypothesis, verifying that the exclusion of sex as a covariate was unlikely to bias estimates obtained from our GWAS (Figure 7).

Results of the fine-mapping analyses for *ARHGAP29*, *SHROOM3*, and *KCNQ5*:

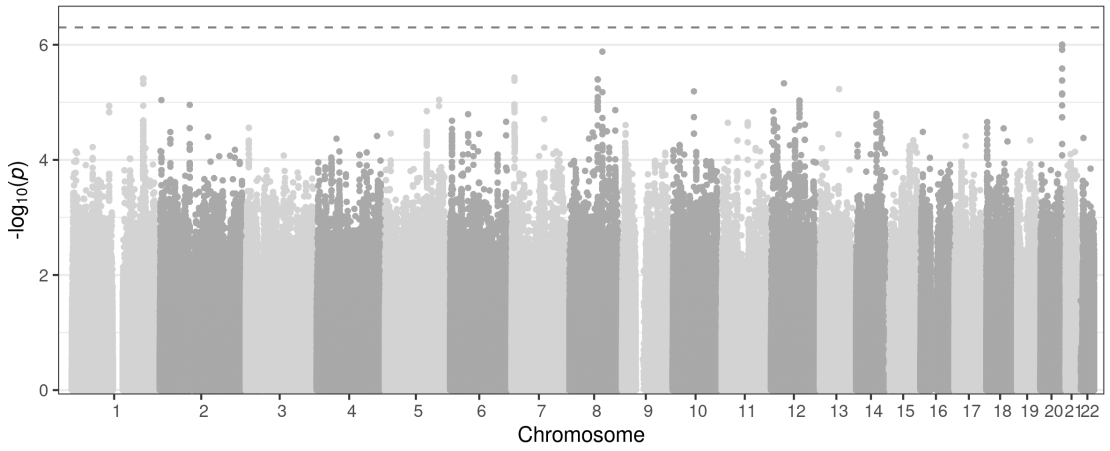

Figure 10: GWAS run using sex as the phenotype. No SNPs met suggestive threshold of $5e-07$ (indicated by the grey dashed line).

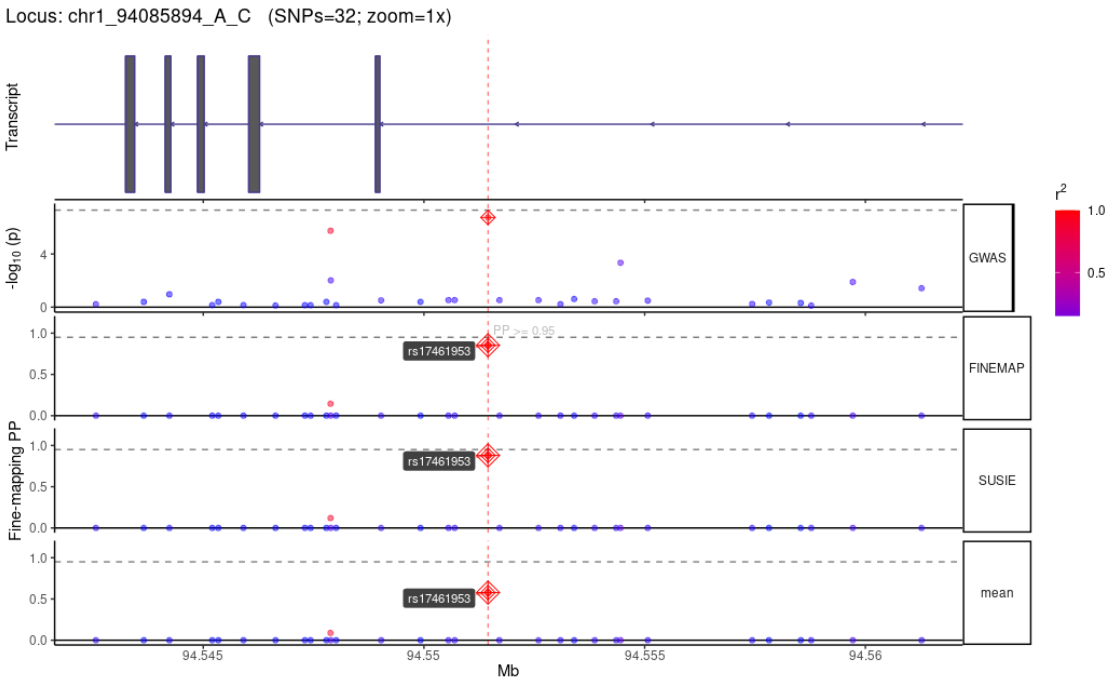

Figure 11: Results of the fine-mapping analyses for the 1Kb region centered on the most significant SNP in *ARHGAP29*.

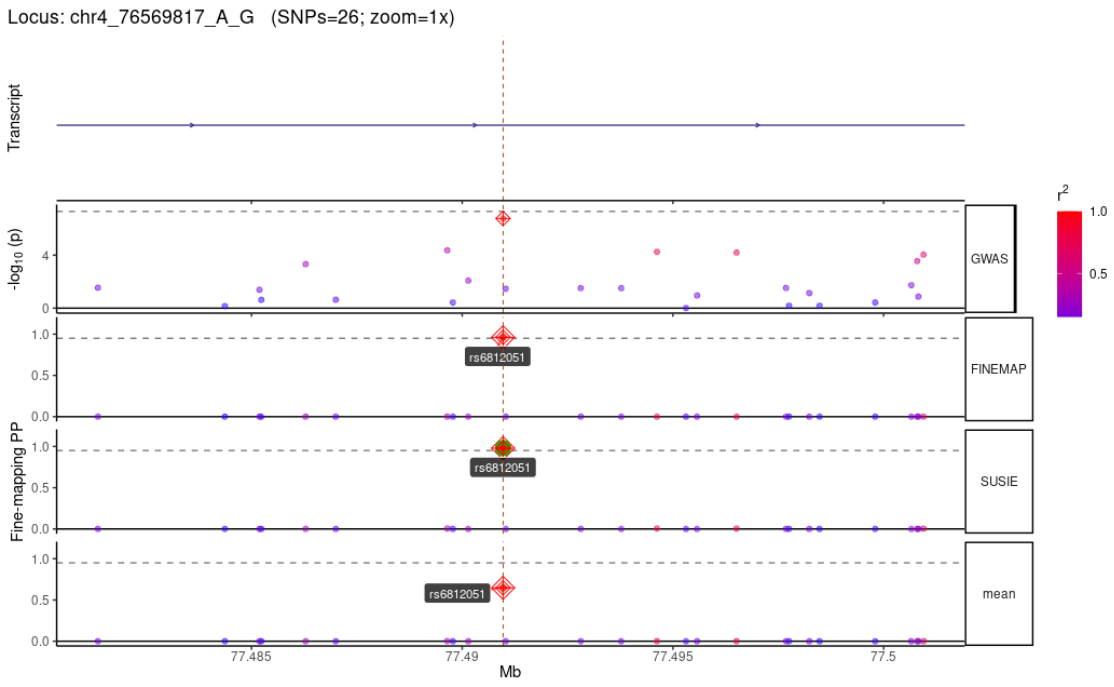

Figure 12: Results of the fine-mapping analyses for the 1Kb region centered on the most significant SNP in *SHROOM3*.

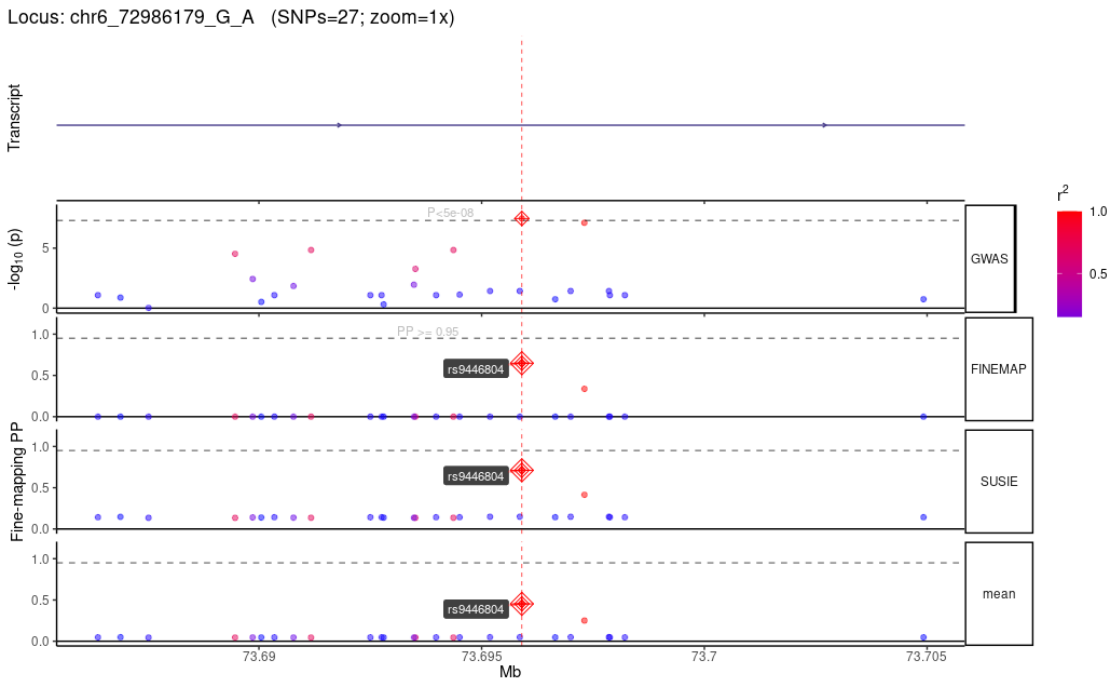

Figure 13: Results of the fine-mapping analyses for the 1Kb region centered on the most significant SNP in *KCNQ5*.

Appendix B Software and Acknowledgements

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Various quality control steps were performed using PLINK (`https://www.cog-genomics.org/plink/`). GWAS, gene-based tests, and COJO analyses were performed with fastGWA-GLMM, mBAT-combo, and COJO respectively, all part of the GCTA software package (`https://github.com/jianyangqt/gcta`). TWAS was performed with S-PrediXcan, part of the MetaXcan software package (`https://github.com/hakyimlab/MetaXcan`). Fine-mapping was performed with the R package echolocatoR (`https://github.com/RajLabMSSM/echolocatoR`).Plots were generated using the R packages geni.plots (`https://github.com/jrs95/geni.plots`), CMplot (`https://github.com/YinLiLin/CMplot`), and ggplot2 (`https://github.com/tidyverse/ggplot2`).

The figure adaptation from Chabora and Horowitz (1974) was created with the online Genogram website (`https://pedigree.progenygenetics.com/`) provided by Progeny Genetics.

Special thanks to Brian Schilder for assistance with the echolocatoR package.

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