Genetic studies of craniosynostosis

with focus on syndromic forms

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Gothenburg 2022

Cover illustration by the author – Schematical cross-section of the developing coronal suture in mouse with DNA fragment

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ISBN 978-91-8009-939-4 (PRINT)
ISBN 978-91-8009-940-0 (PDF)
http://hdl.handle.net/2077/72570

Printed in Borås, Sweden 2022
Printed by Stema Specialtryck AB

To all the patients who help us understand how life takes form

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"He who studies medicine without books sails an uncharted sea, but he who studies medicine without patients does not go to sea at all."

Dr. William Osler

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ABSTRACT

Craniosynostosis (CS) represents the premature closure of skull sutures and affects ~1 in 2500 children. Untreated CS can lead to significant complications in craniofacial and psychomotor development. The only treatment available is surgical and requires access to highly specialized healthcare. The development of alternative and/or complementary therapeutic methods relies on an understanding of the intricate molecular and cellular mechanisms underlying CS. Genetic studies are of clinical importance to establish an etiologic diagnosis of inheritable craniofacial syndromes and enable patient prognosis and follow-up, including assessment of the recurrence risk in the family (genetic counseling).

The aim of this thesis was to study the prevalence and spectrum of genetic alterations associated with CS in a retrospective cohort of patients that underwent surgery at the largest reference center in Sweden. The patients were initially screened with a targeted next-generation sequencing (NGS) panel covering CS-related genes. Patients with negative outcome were subsequently analyzed using whole-genome or whole-exome sequencing (WGS and WES, respectively).

The results showed that targeted NGS screening demonstrated a high diagnostic yield in patients with syndromic forms of CS (>80%) regardless of sutural pattern. The particular case of a patient with coronal synostosis and a Kabuki-like phenotype, as well as a simultaneous *de novo* occurrence of a *lysine-specific methyltransferase 2D (KMT2D)* mutation and 10q22.3q23.1 microdeletion, suggests that CS may be an underdiagnosed feature of these conditions. Additionally, *interleukin-11 receptor subunit α (IL11RA)* was highlighted as an emerging core gene for autosomal recessive pansynostosis. The use of WGS/WES detected causal variants in 38% of the patients with rare syndromic forms of CS and a negative outcome at targeted screening.

Furthermore, potentially relevant variants were observed in 87% of the remaining patients with syndromic or nonsyndromic forms of CS.

These findings showed that both targeted NGS screening and WGS/WES demonstrated a high diagnostic yield in patients with syndromic CS. Moreover, the results suggested that WES/WGS has the potential to become a unique diagnostic tool that can be adapted to the phenotypic presentation by initial use of *in silico* gene panels, followed by exome/genome-wide analysis of rare forms of CS.

Keywords: cranial, suture, genetic, diagnostic, next generation sequencing

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SAMMANFATTNING PÅ SVENSKA

Cirka 1 av 2500 barn födds med kraniosynostos (KS) som innebär en tidig slutning av skallens suturer. Suturerna och fontanellerna består av mjuk vävnad som förbinder skallens ben och är viktiga för skallens tillväxt under det första levnadsåret. KS kan leda till skall- och ansiktsdeformering samt förhöjning av trycket inuti skallen om hjärnan inte har plats att växa vilket kan påverka barnets psykomotoriska utveckling. I nuläget finns enbart kirurgisk behandling som kan förebygga dessa komplikationer.

KS förekommer oftast isolerat, men i vissa fall kan andra missbildningar finnas samtidigt vilket tyder på ett syndrom. Ibland har en av föräldrarna eller andra släktingar haft KS vilket tyder på en ärftlig form. I de flesta fall, särskilt om det handlar om isolerad KS, hittar man inte en säker orsak. Hos ca 25–30% av patienterna har man upptäckt genetiska avvikelser som förklarar förekomsten av KS. Detta gäller särskilt patienter med syndromala former och de som har andra släktingar med KS. Detta projekt hade som syfte att kartlägga de genetiska avvikelser som fanns hos patienter med KS, särskilt hos de med komplicerade former och syndromal bild.

Moderna analysmetoder i form av massiv parallell sekvensering av flera utvalda anlag (gener) kopplade till KS har använts i ett första steg. Detta följdes av helgenomsekvensering (analys av hela arvsmassan) hos de patienter där ingen tydlig genetisk avvikelse kunde hittas vid den första screeningen.

Efter första steget med riktad analys av utvalda gener hittade man en orsaksdiagnos hos mer än 80% av de analyserade patienterna. I de flesta fall handlade om de mest kända syndromen där KS är ett huvudsymptom (t ex Apert och Crouzon syndrom), men man upptäckte mutationer även i mer sällsynta syndrom där KS var ett mindre framträdande symptom (t ex Kabuki och Shprintzen-Goldberg syndrom). Efter andra steget där hela arvsmassan analyserades hos resterande patienter utan diagnos, detekterade man mutationer hos 38% varav många i mindre kända gener för sällsynta syndrom.

Sammanlagt har man visat att med hjälp av moderna analysmetoder så som helgenomsekvensering kan man förbättra diagnostiken hos patienterna med KS. Detta är av stor vikt då en orsaksdiagnos kan påverka behandlingen och ge bättre kunskap om prognosen. Dessutom ger det möjlighet till genetisk vägledning avseende upprepningsrisken i familjen. I framtiden kan man tänka sig att bättre kunskap om den genetiska bakgrunden till KS öppnar vägen till icke-kirurgiska terapier som kan hindra tidig slutning av suturer.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Ţopa A**, Samuelsson L, Lovmar L, Stenman G, Kölby L.

On the significance of craniosynostosis in a case of Kabuki syndrome with a concomitant *KMT2D* mutation and 3.2 Mbp de novo 10q22.3q23.1 deletion. *Am J Med Genet A. 2017 Aug;173(8):2219-2225*.

II. **Ţopa A**, Rohlin A, Andersson MK, Fehr A, Lovmar L, Stenman G, Kölby L.

NGS targeted screening of 100 Scandinavian patients with coronal synostosis. *Am J Med Genet A. 2020 Feb;182(2):348-356.*

III. **Ţopa A**, Rohlin A, Andersson MK, Fehr A, Lovmar L, Stenman G, Kölby L.

The outcome of targeted NGS screening in patients with syndromic forms of sagittal and pansynostosis - IL11RA is an emerging core-gene for pansynostosis. *Eur J Med Genet. 2022 May;65(5):104476.*

IV. **Ţopa A**, Rohlin A, Fehr A, Lovmar L, Stenman G, Tarnow P, Maltese G, Bhatti-Søfteland M, Kölby L.

Genome-wide analysis is an effective diagnostic tool in rare forms of craniosynostosis. *Manuscript.*

CONTENT

ABBREVIATIONS

3D-CT	Three-dimensional computed tomography
ACMG	American College of Medical Genetics and Genom
AD	Autosomal dominant
AR	Autosomal recessive
BMP	Bone morphogenic protein
BSS	Beare–Stevenson cutis gyrata syndrome
cDNA	Complementary DNA
CNCC	Cranial neural crest cell
CNV	Copy number variant
CS	Craniosynostosis
CYP26B1	Cytochrome P450 26B1
DD	Developmental delay
DNA	Deoxyribonucleic acid
EFNB1	Ephrin B1
EN1	Engrailed 1
ERF	ETS-2 repressor factor
ERK	Extracellular signal-regulated kinase
FGFR	Fibroblast growth factor receptor
GLI	Glioma-associated oncogene homolog
gnomAD	The Genome Aggregation Database

HPO	Human Phenotype Ontology
ICP	Intracranial pressure
ID	Intellectual disability
IGF1R	Insulin-like growth factor 1 receptor
IHH	Indian hedgehog
IL11RA	Interleukin-11 receptor subunit α
KMT2D	Lysine-specific methyltransferase 2D
MAPK	Mitogen-activated protein kinase
Mbp	Megabase pair
MLPA	Multiple-ligation-dependent probe amplification
mRNA	Messenger RNA
MSX2	Msh homeobox 2
NCS	Nonsyndromic craniosynostosis
NGS	Next generation sequencing
OMIM	Online Mendelian Inheritance in Man (Catalog of Human Genes and Genetic Disorders)
PCR	Polymerase chain reaction
POR	Cytochrome P450 oxidoreductase
RAB23	Ras-associated binding protein 23
RNA	Ribonucleic acid
RUNX2	Runt-related transcription factor 2
SCS	Syndromic craniosynostosis

SIDS	Sudden infant death syndrome
SMAD6	SMAD family member 6
SNP	Single-nucleotide polymorphism
SPRY	Sprouty RTK signaling antagonist
TCF12	Transcription factor 12
TGF-β	Transforming growth factor-β
TWIST1	Twist family bHLH transcription factor 1
VUS	Variant of uncertain (unknown) clinical significance
WES	Whole-exome- sequencing
WGS	Whole-genome- sequencing
ZIC1	Zinc finger protein of the cerebellum 1

DEFINITIONS IN SHORT

Allele	One of the two or more versions of a DNA sequence (e.g., maternal vs. paternal allele, mutated vs. non-mutated/normal allele).
Amino acid	The building block (molecular unit) of a protein.
Autosomal dominant disorder	Genetic condition caused by a mutation in either the maternal or paternal gene copy situated on an autosome and sufficient to cause symptoms (it 'dominates' the other unmutated gene copy). The risk of transmitting the disorder to offspring is 50% regardless of gender.
Autosomal recessive disorder	Genetic condition that manifests when both the maternal and paternal gene copies (bi-allelic involvement) situated on an autosome pair are mutated. The parents of the individual affected are healthy carriers of the disease while bearing only one mutated gene copy. Their risk of having an affected offspring is 25% regardless of gender.
Autosome	One of two paired chromosomes numbered from 1 to 22. The maternal and paternal chromosomes in each pair are identical in morphology and gene content as opposed to the 23rd pair of sexual chromosomes, which differ in males (XY) and are identical in females (XX).
Base-pair	Two paired nucleotides in the double-stranded DNA helix (Adenine – Thymine, Cytosine – Guanine).

Benign variant	Genetic variant that can affect the structure and/or function of a protein but not expected to cause disease (also called a ‘normal’ variant).
Bicoronal synostosis	Synostosis of both the right and the left coronal sutures.
Brachycephaly	Skull deformation comprising a shortened anteroposterior diameter and a widened interparietal diameter and often the result of bicoronal synostosis.
Chromatin	The complex, compact structure resulting from the wrapping of a DNA molecule around proteins called histones. The chromatin strand folds to form a chromosome.
Codon	A DNA or RNA sequence of three consecutive nucleotides and representing the information unit encoding a specific amino acid in a protein or a stop signal for the translation process (stop/termination codon). One or more codons can encode the same amino acid.
Complementary DNA (cDNA)	DNA synthetized from a single-stranded RNA template using a reverse transcriptase. Used to study gene expression at the mRNA level.
Coronal synostosis	Early closure of the suture separating the parietal and frontal bones. May be uni- or bilateral.
Copy number variation (CNV)	Represents the variation in the quantity of different sections of the genome and may involve parts of a gene, entire genes, or

	larger sections of the genome (e.g., intragenic deletion/duplications, chromosomal microdeletion/duplication).
Cranial suture	Fibrous structures that separate the skull bones.
Craniosynostosis	The early closure of cranial sutures.
Deletion	Loss of genetic material at the DNA-sequence (gene) or chromosome level.
De novo (‘sporadic’)	Refers to a genetic alteration (usually disease-causing) arising for the first time in a family, implying that it was undetected (by analysis of blood cells) in the parents of the affected individual and thus interpreted as a ‘non-inherited’ alteration. However, confined germinal mosaicism (limited to the sperm or eggs) in one of the parents cannot be excluded [(an estimated low recurrence risk in offspring (<1%)].
Deoxyribonucleic acid	Double-stranded helicoidal (spiral-formed) macromolecule situated in the cell nucleus and encoding genetic information. Forms a ‘double-helix’ structure.
Dolichocephaly	Skull shape with a longer-than-expected anteroposterior diameter relative to a narrower biparietal diameter.
Duplication	Excess genetic material at the DNA-sequence (gene) or chromosomal level.
Epigenetic changes	Modifications (reversible) that alter gene expression without changing the DNA sequence. These usually comprise alterations of chromatin (structure of DNA and proteins) as a result of condensation or

	loosening, which restricts or promotes access to the genes for transcription (turns their expression ‘on’ or ‘off’).
Epistasis	Phenomenon by which the expression of a gene and its phenotypic effects are affected by the expression of one or more other genes.
Exome	All exons in the genome.
Exon	Region of a gene that is transcribed into mRNA and often encodes parts of the protein product. There are also noncoding exons.
Frameshift variant	An insertion or deletion of one or several nucleotides (base pairs) that disrupts the triplet (codon) reading frame of a DNA sequence and leads to the premature termination of a protein sequence.
Gain-of-function variant	Genetic variant that either enhances a particular function or creates a new function in the translated protein product.
Genetic (gene) variant	A change in the DNA sequence [‘sequence variant’; e.g., a substitution, loss, or insertion of bases (nucleotides)] or DNA structure (e.g., intragenic deletions or duplications). Pathogenic variants describe a deleterious effect at the protein level that cause disease, whereas benign variants (even when affecting protein structure and/or function) are not expected to cause disease.
Genome	All genetic information of an organism (both coding and noncoding).

Genotype	The genetic make-up of an organism and usually referring to the genetic version (allele/variant) at a certain position in the DNA sequence.
Heterozygous	Individual presenting different maternal and paternal alleles at a specific position in the DNA sequence.
Homozygous	Individual with identical maternal and paternal alleles at a specific position in the DNA sequence.
Intron	Intragenic noncoding regions (between exons) that are removed (spliced) from the primary transcribed (precursor) mRNA (pre-mRNA) to form mature mRNA, which is translated into protein by the ribosome.
Isoforms	Similar proteins with different amino acid sequences but originating from the same gene as a result of alternative splicing. Isoforms may differ in function and present specific tissue- and time(age)-dependent expression.
Lambdoid synostosis	Early closure of the suture separating the parietal and occipital bones. May be uni- or bilateral.
Linkage analysis	Analytical method consisting of a comparison of genetic markers at different positions on the chromosomes in both affected and unaffected family members in order to identify a locus for a disorder which segregates in the family.

Loss-of-function variant	Genetic variant that causes a decrease in or even lack of function of the expressed protein product.
Megabase pair	Unit of length of nucleic acids equal to 1,000,000 base pairs.
Messenger RNA (mRNA)	A type of RNA transcribed from a DNA template (gene) and responsible for transferring the genetic code from nuclear DNA to ribosomes in the cytoplasm, where the mRNA is translated into a protein.
Metopic synostosis	Early closure of the suture separating the frontal bones.
Microarray analysis	Analytical method that detects genome-wide copy number variations, such as loss or gain of genetic material, as well as unbalanced chromosomal aberrations at a higher resolution than conventional chromosome analysis (karyotype). It cannot detect variations at the DNA-sequence level (mutations).
Microdeletion/duplication	Chromosomal deletions/duplications that are too small for detection by conventional chromosome analysis using light microscopy (submicroscopic) and detected by microarray analysis.
Midline synostosis	Synostosis of the metopic and/or sagittal sutures separating the frontal and/or parietal bones, respectively.
Missense variant	Genetic variant that results in a codon that encodes a different amino acid at the protein level. Also referred to as a ‘nonsynonymous variant’.

Modifier gene	Gene in which the occurrence of certain genetic variants may influence (augment or diminish) the phenotypic effects of genetic variants in another gene responsible for the main pathogenic effects. The phenomenon is referred to as ‘epistasis’.
Mosaicism	One or more genetically different cell lines in an individual (e.g., two cell lines with different numbers of chromosomes or in which one cell line harbors a mutated gene). The mosaicism may be confined to one tissue/organ (e.g., gonadal mosaicism affecting the germinal cells in testis or ovaries) or affect some or all tissues of an organism in variable proportions (somatic mosaicism).
Multiple suture (multisuture) synotosis	Pattern of synostosis involving several sutures.
Mutation	A change in the DNA sequence usually associated with disease status. Progressively replaced by ‘pathogenic variant’, which causes a deleterious effect at the protein level.
Next-generation sequencing	Massively parallel sequencing technology enabling the simultaneous analysis of several genes or the entire genome.
Nonsense variant	Genetic variant that leads to a nonsense codon (often a stop codon) that does not encode an amino acid and leads to the premature termination of a protein sequence.

Nonsyndromic craniosynostosis	Craniosynostosis occurring as an isolated event without other developmental abnormalities.
Novel variant	A previously unreported genetic variant.
Nucleotide (‘base’)	One of the four basic molecular units of the DNA macromolecule (adenine – A, guanine – G, cytosine – C, thymine – T).
Pansynostosis	Synostosis of all cranial sutures.
Pathogenic variant	Genetic variant that causes a deleterious effect at the protein level that results in disease. Also referred to as a ‘mutation’.
Phenotype	The observable morphologic and physiologic traits of an organism that result from the expressed genotype in interaction with the environment.
Polymorphism	Genetic variant that is not associated with disease and may lead to variable phenotypic features (such as eye color) in a population. Corresponds to a ‘benign (normal) variant’.
Ribonucleic acid (RNA)	A nucleic acid (usually single-stranded) that contains a sugar moiety (ribose) instead of deoxyribose and includes an uracil base instead of thymine. There are several types of RNAs with different roles in the genetic machinery and with activities in both the nucleus and the cytoplasm.
Sagittal synostosis	Early closure of the suture separating the parietal bones.
Scaphocephaly	‘Boat-like’ skull deformation with an elongated anteroposterior diameter, prominent forehead (frontal bossing), and

	prominent occiput caused by sagittal synostosis.
Segregation analysis	Targeted testing of a genetic variant in affected and/or unaffected family members of an index case in order to explore the genotype–phenotype correlation (in case of a VUS) or whether the variant is *de novo* or inherited.
Sequence variant	Genetic variant that alters the DNA sequence [e.g., a substitution, loss, or insertion of nucleotides (bases) resulting in missense, splice, frameshift, or nonsense variants]. Also referred to as a 'point mutation'.
SNP-array	Type of microarray analysis that targets single-nucleotide polymorphisms (SNPs) at different DNA positions in order to detect CNVs at the genome level.
Splice variant	Genetic variant occurring in the vicinity or at the boundary between exons and introns (splice-site) and affecting mRNA splicing, with loss of exons or retention of introns subsequently altering the protein sequence/structure.
Splicing	The process by which introns are removed from pre-mRNA to form mature mRNA for subsequent translation into protein. Alternative splicing leads to variably spliced mRNA that may retain or remove certain introns and/or skip certain exons and result in different protein isoforms.
Sporadic occurrence	Refers to a genetic alteration that occurred '*de novo*' in a child, implying that it is not

	detected in the parents and thus interpreted as ‘non-inherited’ alteration.
Syndromic craniosynostosis	Craniosynostosis associated with other developmental abnormalities.
Synonymous variant	Genetic variant (nucleotide substitution) that changes the trinucleotide sequence of a codon but not the encoded amino acid. One or more codons may encode the same amino acid; however, depending on their position in the exon sequence, synonymous variants may affect the splicing process.
Synostosis	Ossification of tissue between individual bones, leading to their fusion.
Transcription	The process of creating an RNA copy of DNA.
Transcription factor	Protein that controls the transcription of DNA into RNA following binding to a specific DNA sequence.
Translation	The process by which proteins are synthetized by the ribosome according to the genetic information carried by mRNA.
Trigonocephaly	Keel-shaped deformity of the skull due to early metopic suture fusion, resulting in a triangular shape of the head (as viewed from above).
Truncating variant	A genetic variant that results in an incomplete/short mRNA sequence and translation of shorter version of the protein product (usually nonfunctional).
Turricephaly	Skull deformation comprising a tall, ‘tower-like’ head and often resulting from

	combined coronal and sagittal synostosis. Also referred to as ‘acrocephaly’ or ‘oxycephaly’.
Variant of uncertain clinical significance (VUS)	Genetic variant which, due to its molecular characterization with uncertain effects at the protein level, lack of previous reports, and uncertain phenotypic correlation, cannot be classified as either likely benign or likely pathogenic.
X-linked	Related to the X chromosome (e.g., X-linked disease, which is caused by a mutation in a gene on the X chromosome and that usually affects males more than females, who have two X-chromosomes).
Whole-exome sequencing	The analysis of all exons (the exome) in a genome and essentially representing the protein-coding regions.
Whole-genome sequencing	The analysis of all genetic information in an organism (both coding and noncoding regions).

1 INTRODUCTION

This study straddles the border between fundamental research and applied clinical science. This particular research field belongs to a larger domain best defined as developmental biology, which focuses on understanding the mechanisms that govern the development of different organisms. The genetic code, the foundations of which are shared between species, plays a central role. Genes can be broadly compared to books in a cellular library, as the translation of their information into proteins in specific spatiotemporal order determines the role (function) of different cell lines and their organization into tissues, body parts, and organs. This process can be affected by intrinsic and extrinsic factors in the embryo. The genetic (intrinsic) factors, represented by different types of changes in the DNA structure (generally known as mutations), often cause disturbances of the developmental process that lead to various abnormalities that sometimes follow a defined pattern (a syndromic presentation) depending on the role of the affected gene. Extrinsic factors can include toxic (teratogenic) substances, including drugs, gamma radiation, or other environmental factors.

This thesis focused on the genetics of craniosynostosis (CS), which describes the premature closure by ossification (synostosis) of one or several skull sutures and can result in potentially severe complications in the form of neurological impairments and craniofacial deformation. The cranial sutures are fibrous structures that separate the cranial bones and allow the growth of the skull in relation to the developing brain. The sutures also enable reversible deformation of the head in the birth canal and temper the mechanical stress supported by the skull. The expansion of the human brain is a particularly rapid process during the fetal and infantile periods and requires sutural patency. CS can be detected during the second trimester of pregnancy due to an abnormal head shape that is usually observable at birth. Postnatal suture fusion occurs less frequently and may be underdiagnosed because of its insidious development. CS can occur as an isolated event corresponding to a nonsyndromic presentation or in combination with other malformations and/or neurocognitive issues, thereby representing a syndromic form. Isolated, nonsyndromic CS (NCS) is observed in ~70% of cases, with the remaining presenting syndromic forms (SCS). However, the distinction between NCS and SCS can be difficult, especially in infants, in whom the detection of other developmental abnormalities, including neurocognition, may be delayed.

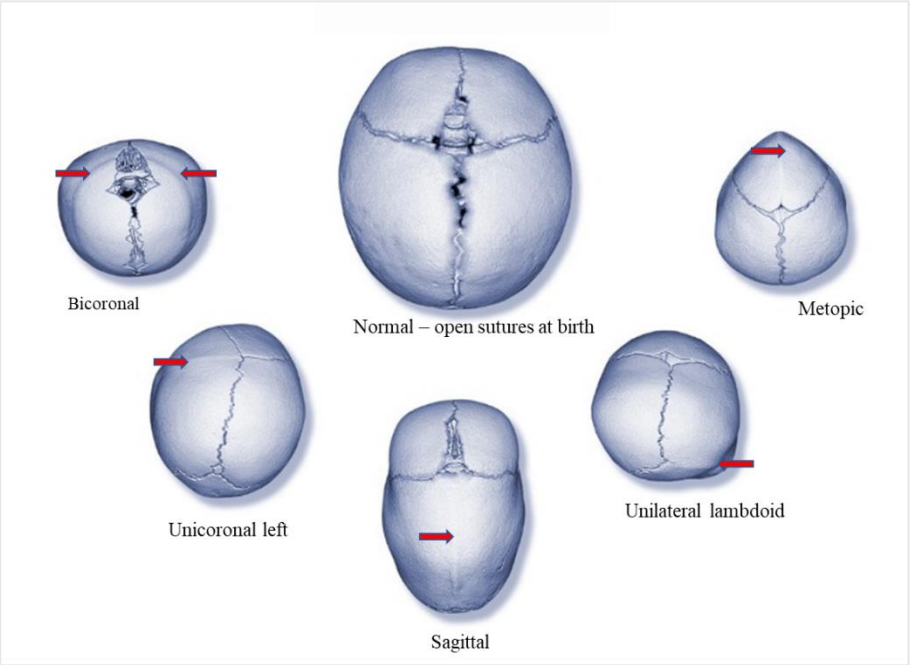

Figure 1. Types of CS according to the synostotic suture. Illustration by Niclas Löfgren.

Premature closure of a suture leads to restricted skull growth in a direction perpendicular to the synostotic suture. Depending on which suture(s) is involved, CS is classified as sagittal, coronal, metopic, lambdoid, or multiple (**Figure 1**). Sagittal NCS is the most frequently observed form of synostosis (45–58% of cases), whereas coronal synostosis or its combination with other suture synostoses is dominant among forms of SCS [7, 8].

The worldwide prevalence of CS is difficult to estimate due to a lack of systematic studies in certain regions. According to a recent meta-analysis of publications from different parts of the world, the overall birth prevalence was ~6 cases per 10,000 live births, with NCS observed in ~5.2 cases per 10,000 live births, in 2019 [9]. In Sweden, the epidemiology of CS was recently updated to an incidence of ~7.7 cases per 10,000 live births, with SCS observed in ~0.60 in 10,000 cases. For NCS forms, 58% of cases were represented by sagittal CS and 25% by metopic CS [10]. Notably, an increase in the incidence of isolated single-suture metopic synostosis and even sagittal synostosis has been observed in different studies but without a clear explanation, although improved diagnostic and environmental factors have been discussed [10-12].

The only treatment for CS is surgery in order to prevent increases in intracranial pressure (ICP) and skull deformation. Diagnosis is performed upon physical examination and confirmed by three-dimensional computed tomography (3D-CT) reconstruction. Children with CS undergo extensive surgical interventions during their first year of life. Among the most frequent and serious complications in these patients is intraoperative bleeding requiring transfusion in ~80% of cases [13].

CS is caused by the disruption of normal cell migration, proliferation, and differentiation in skull tissues. Although the pathogenetic process is not completely understood, especially in NCS forms, it is believed to be heterogeneous, comprising a complex interplay between potential intrauterine environmental factors, brain development, and genetic factors. However, the situation differs in SCS forms, where genetic factors play a dominant role.

In recent decades, mutations in specific genes (monogenic causes) and chromosomal abnormalities have been described in relation to CS. Chromosomal anomalies, such as unbalanced translocations or microdeletion/duplications, have been detected in up to 15% of SCS cases [14]. Notably, a monogenic cause was identified in ~69% of these cases but only 5% of NCS cases [15]. As a result, NCS is believed to have a multifactorial etiology rather than an exclusively genetic background. However, recent results from genome-wide association studies, whole-exome sequencing (WES), and twin studies support the hypothesis of a significant implication of genetic factors, even in NCS [16-18].

Additional knowledge regarding the genetic factors involved in CS will have clinical implications in terms of both diagnostics, with access to genetic counselling to determine recurrence risk in families, and future therapeutic strategies.

1.1 FROM CLINICAL TO MOLECULAR DIAGNOSIS

Craniofacial appearance influences how individuals and groups identify themselves and socialize. Anthropologic studies on skeletal remains and art objects reveal that intentional cranial deformation using different techniques to attain a desired head shape in infants has been widespread in both ancient and even more recent cultures worldwide. The aims of such alterations vary and are often related to the attainment of cultural aspects of beauty or appearances associated with warriors or aristocrats. Unintentional deformation and possible CS were identified by studying skulls from different time periods, with the oldest being at least 500,000 years from the Middle Pleistocene and harboring signs of unilateral lambdoid synostosis and evidence of elevated ICP. Interestingly, depictions of the Egyptian pharaoh Akhenaten (~1350 B.C.) suggest the possible presence of a form of CS associated with androgynous features similar to those of Antley–Bixler syndrome. The first systematic descriptions of CS are attributed to Hippocrates in the ~4th century B.C. Hundreds of years later, the Roman encyclopedist Cornelius described skulls without sutures, and in the 16th century A.D., the anatomist Andreas Vesalius from Brussels noted different types of skull deformities. In the late 18th century, Samuel Thomas Sömmering, a German physician and anatomist, identified the sutures as sites of early skull growth and associated premature suture fusion with cranial deformation. The term ‘craniostenosis’ was coined by Rudolph Virchow, a German scientist and physician, who in 1851 proposed a classification of deformities caused by single-suture fusion and described the mechanism of restricted growth in a direction perpendicular to the synostotic suture with compensatory overgrowth along the suture. Notably, Virchow made no clear distinction between a small head due to osteogenic factors and microcephaly due to insufficient brain growth with secondary CS, which is essential for patient diagnosis and treatment [19, 20].

Another important distinction in diagnosis of cranial deformation is between CS and deformational cranial flattening without sutural fusion. The latter occurs frequently in infants with preferential positions of the head against a solid surface, which creates an occipital flattening that can be either symmetric [leading to deformational brachycephaly (from the Greek ‘brachy’, meaning ‘short’, and ‘kephale’. meaning ‘head’] with compensatory parietal widening or asymmetric [as in deformational plagiocephaly (from the Greek ‘plagio’. meaning ‘oblique’)] with some degree of ipsilateral frontal protrusion (**Figure 2**). Viewed from above, the head shape in deformational plagiocephaly

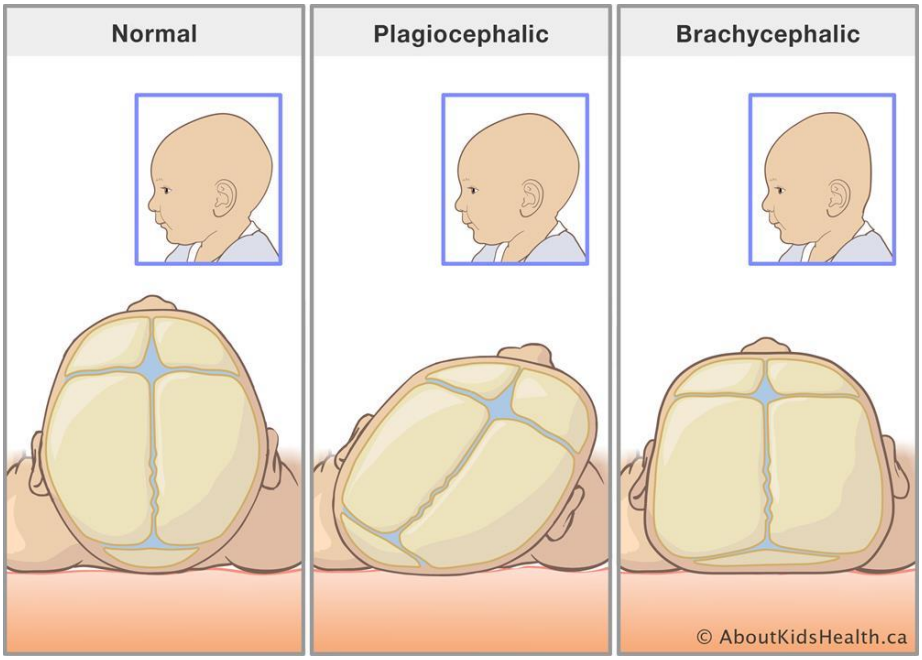

Figure 2. *Deformational cranial flattening. Illustration reproduced from © 2004-2022 AboutKidsHealth.*

resembles a 'parallelogram' [21]. These represent the most frequent skull deformations, with an estimated prevalence of ~20% in healthy infants. In the United States, the high prevalence was considered a consequence of the "Back to Sleep Campaign" promoted in 1992 by the American Academy of Pediatrics and based on evidence that a supine position (on the back) during sleep might prevent sudden infant death syndrome (SIDS). Wide implementation led to a 40% reduction in SIDS incidence in North America and a simultaneous exponential increase in observed deformational/positional plagio- or brachycephaly [22]. However, there is no clear cause and effect correlation, as the majority of supine-positioned infants do not develop skull deformation. It is hypothesized that sleeping on the back amplifies the effect of other predisposing factors to cranial deformation, such as limited head mobility (as in congenital muscular torticollis) or deficient bone mineralization (as in osteogenesis imperfecta). Positional plagiocephaly needs to be distinguished from unilateral coronal synostosis and lambdoid synostosis, which are comparably rare relative to deformational flattening. Unilateral coronal synostosis leads to anterior plagiocephaly, which includes alteration of the orbital rim with orbital protrusion, deviation of the nasal root and midface, and compensatory contralateral frontal protrusion with asymmetric orbital rims. The difference between posterior plagiocephaly and lambdoid synostosis is

more difficult to detect and includes a shorter cranial height with asymmetric positioning of the ears in synostosis versus to the increased cranial height on the flattened side present in deformational plagiocephaly. In deformational brachycephaly, the posterior vertex may be taller than the frontal region, resulting in a sloped appearance to the head as viewed in profile. Brachycephaly as a consequence of bicoronal synostosis is distinguished by forehead retrusion, including the orbital rims, which leads to eye protrusion (proptosis) and anterior turricephaly (a 'tower-shaped' forehead). Deformational scaphocephaly (a 'boat-like' skull) is rare but more frequently observed in infants with exaggerated head rotation or in premature infants positioned side-to-side in the intensive care units, which results in facial asymmetry due to asymmetric growth on the flattened side. This is not the case in scaphocephaly caused by sagittal synostosis, which results in frontal bossing and decreased height of the posterior skull with a prominent occiput [21].

Due to its special craniofacial features, associated malformations, and often disrupted neuropsychological development, SCS has received increased attention. Certain patterns of abnormalities combined with dysmorphic features result in clinical diagnostic criteria that define specific syndromes (from the Greek 'syndromon', meaning 'concurrence'). The study of different malformation patterns and their diagnoses, etiology, and classification is the object of dysmorphology (syndromology), which originated in Europe at the beginning of the 20th century. Most congenital malformation syndromes were first noticed by pediatricians. In 1906, the French pediatrician Eugène Charles Apert described a patient with acrocephaly (other term for turricephaly) and syndactyly of the hands and feet, recalling that similar cases had been described at the end of the 19th century. He subsequently referred to the syndrome as acrocephalopolysyndactyly [20]. The etiology of the Apert syndrome was discovered 100 years later by Wilkie et al. [23] using a positional candidate gene approach according to evidence of specific mutations in *fibroblast growth factor receptor* (*FGFR*)*2*. A few years later, Louis Edouard Octave Crouzon, a French neurologist specializing in hereditary conditions, described the case of a mother and son with similar features of craniofacial dysostosis and continued to study families with the same syndromic presentation, which was later named after him. Mutations in the same gene (*FGFR2*) were identified in 1994 by linkage analysis[24]. Both the Norwegian neurologist and psychiatrist Haakon Saethre and the German psychiatrist Fritz Chotzen described patients with a familial form of turricephaly that included signs of increased ICP and other limb abnormalities (e.g., skin syndactyly of the hands and feet), with *Twist family bHLH transcription factor 1* (*TWIST1*) later identified as the causal gene for Saethre–Chotzen syndrome in 1997 [25, 26]. The German geneticist Rudolph Pfeiffer

described several members of a family with acrocephaly and broad thumbs and great toes as a resident in pediatrics in 1964. Almost 30 years later, in 1991, Maximilian Muenke, a clinical geneticist, studied the same family and collected samples for linkage analysis. Muenke et al. [27] in 1994 and others in 1995 [28-30] showed that Pfeiffer syndrome was caused by a recurrent mutation in *FGFR1* and mutations in *FGFR2*. Shortly thereafter, in 1996, a recurrent mutation in *FGFR3* was detected in families initially considered to have Pfeiffer syndrome because of broad halluces but with other distinct features, such as thimble-like middle phalanges, coned epiphyses, and carpal and tarsal fusions. The findings suggested that recurrent mutations in analogous positions within FGFR1 (p.Pro252Arg), FGFR2 (p.Pro253Arg), and FGFR3 (p.Pro250Arg) led to the partially overlapping phenotype but distinct clinical syndromes, such as Pfeiffer, Apert, and Muenke, respectively [31, 32]. Additionally, the same mutations in *FGFR2* could lead to different phenotypes, such as Pfeiffer and Crouzon [29]. This marked the transition from a classification based on clinical criteria to molecular characterization, which revealed the pathogenic mechanisms underlying related syndromes.

In recent decades, the development of high-throughput (massively parallel) sequencing techniques enabled the discovery of several other core genes involved with CS, as well as novel genotype–phenotype associations.

David Weyhe Smith, an American pediatrician and clinical geneticist (also known as 'the father of dysmorphology') contributed to the education of clinical geneticists and pediatricians by expanding the understanding of the etiology, mechanisms, and diagnosis of congenital anomalies through his book, entitled '*Recognizable Patterns of Human Malformation'*, published in 1970. Smith emphasized the importance of distinguishing a deformational sequence secondary to external forces from a malformation supposing a primary cause. A subsequent book, entitled '*Recognizable Patterns of Human Deformations*', was published posthumously in 1981. In his introduction, Smith mentions the British pediatrician Peter Dunn, a pioneer of perinatal medicine and the source of inspiration for Smith's study of human deformations [33]. Notably, Dunn and Smith supported the hypothesis of fetal constraint as a cause of sagittal synostosis and proposed its classification as a congenital postural deformity [34].

1.2 THE MOLECULAR DIAGNOSTIC ODYSSEY

Modern genetics began with publication of Gregor Johann Mendel's memoir on plant hybridization in 1866. Mendel, a German-speaking biologist from the Silesian region of the Austrian Empire (today's Czech Republic), experimented with cross-breeding of different types of pea plants and observed the distribution ratio of features in offspring. Mendel's intention was not to formulate general laws of heredity but propose a 'law of the development of hybrids' in plants. In 1902, the British biologist William Bateson published results from studies on both plants and animals that supported Mendel's 'law of segregation applying to just one character'. Bateson introduced technical terms, such as 'allele', 'homozygote', and 'heterozygote', with reference to these characters and implied that for a given character inherited in a Mendelian way, an individual has two physical versions of the same hereditary element. Genetics as a discipline was officially created in 1906 after a meeting of the international community involved in heredity studies. Wilhelm Johannsen, a Danish pharmacist and botanist, proposed the term 'gene' for the 'calculating unit' (the hereditary element) intervening in Mendelian crosses, although in the absence of a hypothesis regarding the nature of this element. The term was inspired by another term used by the Dutch botanist Hugo de Vries in his book from 1889 (*Intracellular Pangenesis*) to describe the hereditary particles in all cells of an organism: 'pangene'. Simultaneously, cytologists continued to accumulate knowledge concerning the morphology of chromosomes during meiotic and mitotic cell division. Walter Sutton, an American geneticist, and Theodor Boveri, a German zoologist and anatomist, proposed the chromosomes as bearers of hereditary Mendelian factors. This hypothesis was initially contested by both Bateson and Thomas Hunt Morgan, an American evolutionary biologist and geneticist, who eventually published his work on heredity with *Drosophila melanogaster* (fruit fly) in 1915 together with three other colleagues (*The Mechanism of Mendelian Heredity*). The results of their work supported the correlation between the chromosome and the Mendelian factors ('genes'), the latter being part of the chromosomes. This was a fundamental step in the understanding of the genetic mechanisms of heredity. From this point forward, the concept of genes as part of a chromosome with a specific location solidified, although, the nature of the genes remained elusive. In 1953, Francis Crick and James Watson together with the British biophysicist Maurice Wilkins determined the helical structure of the DNA molecule and for which they were awarded the Nobel Prize in Physiology or Medicine in 1962 [35]. Work on the DNA structure by the British chemist and X-ray crystallographer Rosalind Franklin was an indispensable contribution to this

discovery [36]. Crick and colleagues continued their work during the 1950s on deciphering the genetic code and how the four nucleotides or bases (adenine - A, thymine - T, cytosine - C, and guanine - G) encode instructions for assembling the twenty amino acids into proteins. They launched the concept of the 'codon' as a three-base combination that corresponds to a specific amino acid or translation 'stop' signal. Shortly after the discovery of the DNA structure, experimental work on bacteria by the French scientists François Jacob (biologist) and Jacques Monod (biochemist) demonstrated the role of DNA transcriptional regulation on enzymatic expression, thereby paving the way for an emerging scientific field represented by molecular developmental biology. Together with the French microbiologist André Lwoff, they received the Nobel Prize in Physiology or Medicine in 1965. Three years later, the Americans Marshall Nirenberg, Har Gobind Khorana (Indian American), and Robert Holley were awarded the Nobel Prize in Physiology or Medicine for their contribution to interpreting the genetic code and how it is translated into proteins, in particular the role of messenger RNA (mRNA) as an intermediary molecule between the DNA template and the protein, as well as the transfer RNAs involved in construction of the protein sequence. During the same period in the 1950s, discoveries of ribosomes and their role in protein synthesis by the Romanian American cell biologist George Emil Palade led to a Nobel Prize in Physiology or Medicine in 1974 together with the Belgian American cell biologist Albert Claude and the Belgian cytologist and biochemist Christian de Duve.

In 1955, the number of chromosomes in humans was determined by the cytogeneticists Joe-Hin Tjio and Albert Levan working together at the Institute of Genetics in Lund, Sweden. Tjio sent photographic copies of the discovery to friends and colleagues around the world with the note: 'Human cell with 46 chromosomes observed 1955 on December 22nd at 2.00 am' [37]. The 46 chromosomes are grouped in 23 pairs, with each pair containing a maternal and paternal chromosome representing a diploid set and characteristic of somatic (bodily) cells, whereas sperm and eggs (germinal cells) contain 23 chromosomes representing a haploid set. The chromosome pairs (numbered 1–22) are called autosomes, with the maternal and paternal chromosomes being identical in morphology and gene content. The 23rd pair of chromosomes represents the sex chromosomes, which are identical in women (conventionally denoted as XX) but different in men (conventionally denoted as XY). The Y chromosome contains a significantly smaller number of genes than the X chromosome; however, genes unique to the Y chromosome include *sex(testis)-determining region Y*. This explains why males are usually more affected by mutations in genes on the X chromosome than females, who have two copies of the respective genes. One of the X chromosomes in females is

randomly inactivated (a process referred to as 'lyonization' after the British geneticist Mary Frances Lyon), leading to a mosaic cellular state, in which either the maternal or the paternal X chromosome is active. As a result, skewed inactivation of a healthy X chromosome in favor of an X chromosome bearing a mutated gene can lead to symptoms of an X-linked disorder in females.

In 1977, the British molecular biologist Richard Roberts and the American geneticist Phillip Sharp discovered the existence of 'split genes' and 'introns' as noncoding regions of genes (**Figure 3**) that are removed (spliced) from precursor mRNA (transcribed from the DNA template), which subsequently becomes mature mRNA containing only the transcribed sequence of exons that encode the protein sequence translated by ribosomes. Alternative splicing can lead to different protein variants (isoforms) in terms of amino acid sequence. These isoforms either lack or include regions depending on which exons are spliced or retained in the genetic sequence and the subsequently transcribed mRNA sequence. Isoforms of the same protein can differ in function and demonstrate tissue-specific and age-dependent expression. Roberts and Sharp

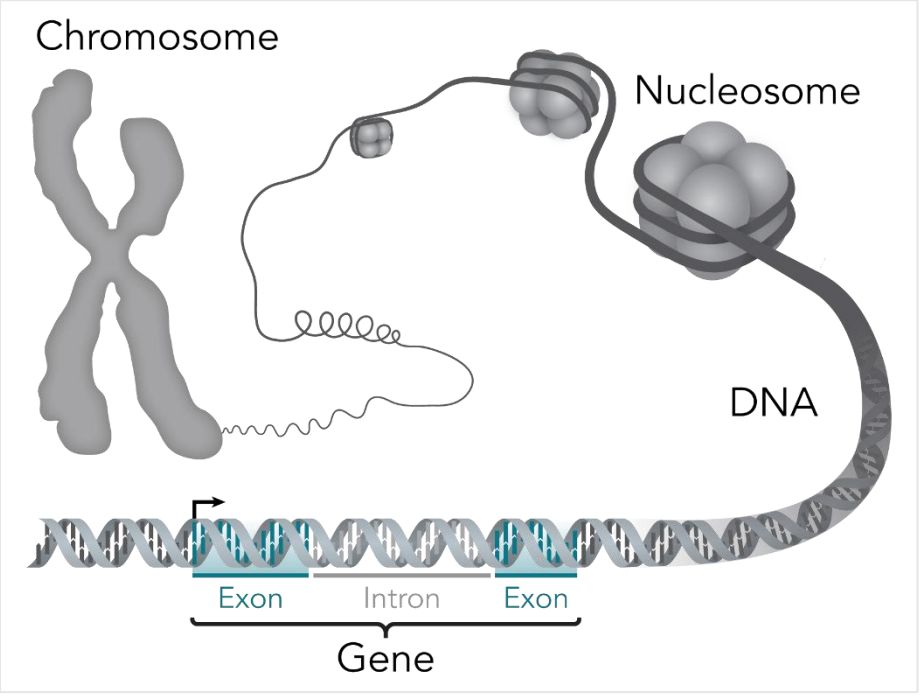

Figure 3. Representation of a chromosome including the chromatin thread wrapping around proteins called histones (spherical form) to form a nucleosome. DNA thread with representation of the exonic and intronic regions of a gene. Illustration by Thomas Splettstoesser from Wikimedia Commons.

received the Nobel Prize in Physiology or Medicine in 1993, the same year that Karry Mullis, an American biochemist, received the Nobel Prize in Chemistry for his invention of the polymerase chain reaction (PCR) in 1985. This technique fundamentally changed the field of molecular biology and medical genetics by enabling amplification of specific DNA regions. Before the invention of PCR, the first DNA-sequencing technique was developed by the British biochemist Frederick Sanger (whose name was given to the method) and the American biochemist Walter Gilbert, who received the Nobel Prize in Chemistry in 1980. Notably, Sanger had also received the prize in 1958 for his work on determining the structure of multiple proteins, including insulin. These methods enabled the mapping of gene loci at the chromosome level and subsequent identification of genes encoding previously known proteins. Importantly, the role of these proteins in different human disorders either remained unknown or was assumed based on studies of animal models, which generated candidate genes based on a phenotype-to-genotype approach.

Identification of the first mutated gene in CS occurred in a family with autosomal dominant CS using linkage analysis to map the locus of *Msh homeobox 2* (*MSX2*), which encodes a transcription factor harboring homeodomains implicated in the regulation of other genes typically involved in embryonic development. Studies in mice showed that *Msx2* was expressed in neural crest-derived mesenchyme and osteogenic tissues. Linkage analysis in this family specifically identified a locus on the long arm of chromosome 5 (5qter region). After establishing linkage between *MSX2* and the 5qter locus, the mutation was discovered by sequencing *MSX2* [38]. Similarly, mutations in other CS-related genes, such as *FGFR2*, *FGFR3*, and *TWIST1*, were subsequently identified. Another method for discovering candidate genes involves evaluating the breakpoints of chromosomal translocations identified in patients with a syndromic presentation. This method was applied for *glioma-associated oncogene homolog* (*GLI*)*3*, in which an interruption was detected at chromosomal breakpoint 7p13 in families with Greig cephalopolysyndactyly syndrome [39].

The Human Genome Project was launched in October 1990 and completed in April 2003, having achieved the goal to map and sequence all of the genes in the human genome. The project revealed the importance of noncoding regions, which had previously been regarded as 'junk DNA' and represent ~98% of the genome, whereas the remaining regions comprise sequences that encode proteins. Noncoding regions play diverse roles in regulating the expression of coding regions, as well as encoding different types of regulatory RNAs.

Among the greatest ongoing challenges in modern genetics is understanding the involvement of these regions in human pathology. Furthermore, there remain unmapped regions of the genome with unknown functions.

Identification of disease-associated genes has motivated further research related to human genetics and the development of analytical methods for the molecular diagnosis of diseases. However, these techniques were often costly, required highly specialized personnel, and were time-consuming. In the case of CS, PCR and Sanger sequencing used to target specific mutations in genes known to cause the classical phenotypes. This resulted in an inability to identify mutations in other genetic regions as in the case of patients with non-classical presentations of SCS, who might have harbored mutations in as yet unidentified genes. However, rapid progress in diagnostic techniques and increased knowledge regarding the genetic and phenotypic heterogeneity of diseases expanded the regions of the genome targeted for analysis and testing strategies with stepwise approaches were developed. This included initially focusing on mutational hot spots, followed by expanded assessment of other exonic regions of a gene as well as other genes depending on the patient's phenotype. Furthermore, these sequencing techniques detected only point mutations; therefore multiple-ligation-dependent probe amplification (MLPA) was developed to allow detection of other types of mutations, such as deletions or duplications of larger genetic regions.

Conventional karyotyping (chromosomal analysis) using light microscopy represented the standard first-tier analysis in cases of syndromic presentations according to its ability to detect large chromosomal aberrations (e.g., unbalanced translocations, partial deletions, and trisomies). Additionally, targeted analysis of specific chromosomal regions known to be implicated in microdeletion syndromes, such as 22q11, was performed using fluorescence *in situ* hybridization (FISH). In the previous decade, cytogenetic methods were replaced with array-based techniques with higher resolution, such as single-nucleotide polymorphism (SNP)-microarray as a method of quantitative analysis using probes that cover the majority of genomic regions and able to detect both larger chromosomal imbalances and smaller (submicroscopic) microdeletions/duplications. These array-based techniques are currently used for first-tier analysis in cases of syndromic presentations without suspected diagnoses or in cases with several possible differential diagnoses, including chromosomal abnormalities. However, patients with classic syndromic phenotypes, which represent the majority of SCS cases, are usually screened for mutations in known CS-specific genes (*FGFR1/2/3, TWIST1,* and *MSX2*). Other CS-related genes, such as *ephrin* (*EFN*)*B1* (craniofrontonasal dysplasia)

and *cytochrome P450 oxidoreductase* (*POR*; Antley–Bixler with genital anomalies), were discovered at the beginning of the 21st century [40, 41].

This stepwise approach was progressively replaced by next-generation sequencing (NGS), which enabled the rapid, accurate, and simultaneous analysis of several genes with high efficacy and lower costs than previous approaches. CS-specific genes such as *ETS-2 repressor factor* (*ERF*) and *transcription factor 12* (*TCF12*), both discovered using NGS, were subsequently included in diagnostic panels [42, 43].

Subsequent introduction of whole-exome and whole-genome sequencing (WES/WGS) enabled multiple breakthroughs, including the discovery of new genes explaining many human disorders. WES analysis targets all known protein-coding regions (exons) in the genome, whereas WGS evaluates both coding and noncoding regions. During the last years, these state-of-the-art technologies have been introduced in the diagnostic routine of many clinical labs around the world. WES/WGS enable *in silico* targeted analysis of bioinformatically selected genes from the whole-exome/genome data. In case of negative outcome, the analysis can be extended to the rest of the data in a so-called exome/genome-wide analysis especially in case of syndromic presentations. Parental samples can be added to the index-case analysis (in a so-called Trio or Quadro analysis in case of two affected siblings) to provide additional information concerning the inheritance pattern, thereby improving the interpretation of previously unreported variants. WGS has the advantage of a better uniformity of coverage over different regions of the genome, thus improving the detection of both sequence variants and copy number variations (CNVs) and precluding the need for MLPA analysis The disadvantages of WGS are the associated costs and the large amount of data provided, which is challenging in terms of storage and interpretation. As a result, WES is often preferred; however, this method only allows analysis of 1% to 2% of the genome [44].

At the Department of Clinical Genetics and Genomics, Sahlgrenska University Hospital, Gothenburg, Sweden, targeted sequencing of *TWIST1/FGFR2/FGFR3* using the Sanger technique with complementary MLPA analysis was performed until 2014, when a targeted NGS panel covering 12 CS-related genes was introduced. The NGS-panel was expanded to cover 29 genes in 2018, after which WGS was introduced in 2019 following validation of an *in silico* panel of 33 CS-related genes, which is now applied for first tier screening of patients with CS. MLPA was maintained as complementary analysis for genes with reported CNVs.

1.3 PATHOPHYSIOLOGICAL ASPECTS OF THE CRANIAL SUTURE DEVELOPMENT

Craniofacial development is a complex process that requires fine tuning between cell proliferation, migration, and differentiation. The process is governed by the spatiotemporal activity of different transcription factors and growth factors involved in signaling pathways that determine the fate of different cells and eventually shape the head structures.

An understanding of the normal and pathological development of cranial sutures in humans is hampered by the complexity of the associated processes; however, accessibility to animal models, such as rodents (mouse and rat), rabbit, sheep, frog, and zebrafish, enables relevant comparisons. These studies have demonstrated the existence of evolutionarily conserved signaling pathways that, when disturbed, lead to similar abnormalities in humans [45].

The calvarial bones (two frontal and two parietal) are formed by intramembranous ossification that differs from endochondral ossification, during which a preexisting cartilaginous structure is ossified (occipital bone and skull base). During embryonal development, a group of multipotent cells originating from the neural crest [cranial neural crest cells (CNCCs)] undergo epithelial-to-mesenchymal transition and begin migrating toward the future sites of craniofacial structures. At the ~5th week of gestation, the craniofacial mesenchyme mostly comprises CNCCs along with the paraxial mesoderm. The parietal and occipital bones are of mesodermal origin, whereas the frontal bones are of ectodermal (neural crest) origin. The cranial bones are formed by the condensation of mesenchymal cells forming ossification centers, from which mineralization starts to propagate radially at the ~13th week of gestation. By the 18th week of gestation, the osteogenic fronts approach, with the sutures formed in between. The skull bones continue to expand along with brain development by appositional growth at the sutural margins [46-48]. The metopic is the only calvarial suture which closes in infancy (between 6 and 12 months of age), whereas the other calvarial sutures (coronal, sagittal, and lambdoid) close in adulthood in the third decade of life. Notably, the sutures of the face skeleton remain open until the 7th or 8th decades [7, 49].

As reviewed by Twigg and Wilkie [50], five spatiotemporally overlapping processes can be distinguished during the genesis of cranial sutures. The initial process comprises ***stem cell specification and migration***, which has been demonstrated in studies of mice, in which cells expressing *Gli1* [a marker of

Sonic hedgehog signaling (SHH) – **Figure 4**] from the paraxial mesoderm migrated toward a region above the developing eye, where other migrating cells from the neural crest concentrated to form the supraorbital regulatory

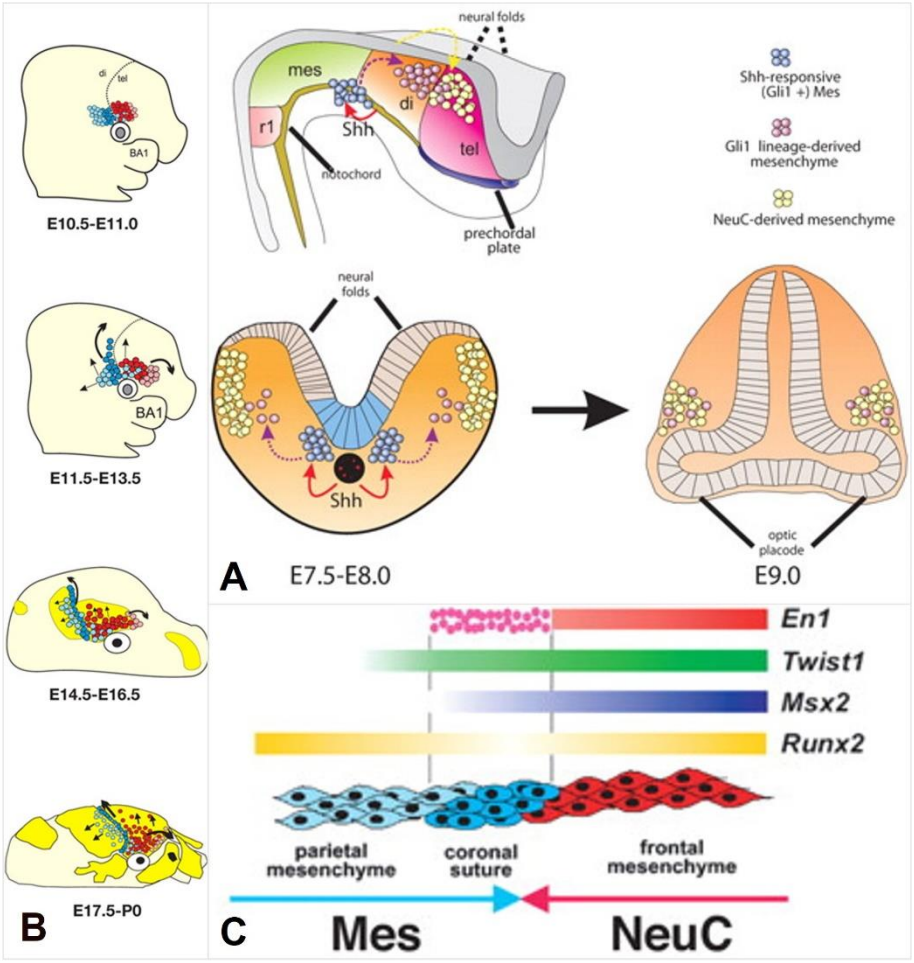

Figure 4. Cranial suture development in mouse. ***(A)*** *Role of SHH-signaling and Gli1 in the migration of cells from the paraxial mesoderm. r1-first rhombomere, mes-mesencephalon, di-diencephalon, tel-telencephalon, E-embryonic days.* ***(B)*** *Coronal suture and adjacent bone formation by migration of different cell populations expressing En1 from the supraorbital domain: mesenchymal (Mes)-derived coronal suture (blue), parietal and frontal bone progenitors (light blue), and neural crest (NeuC)-derived frontal bone progenitors (red and pink). BA1-first branchial arch.* ***(C)*** *Spatial relationship between the expression of different transcription factors in the cell populations of the supraorbital regulatory center. Compiled illustrations from Deckelbaum, Holmes [5](https://doi.org/10.1242/dev.076729) used with permission from The Company of Biologists Ltd., conveyed through Copyright Clearance Center, Inc. (License ID1276196-1).*

center. Thereafter, the cells continue to migrate and populate the coronal sutures, parietal, and frontal bones. The coronal, sagittal, and lambdoid sutures are mostly of mesodermal origin with minor ectodermal contribution from the neural crest, whereas the metopic suture, the frontal bones, and the dura mater are of neural crest origin. *Gli1*-expressing cells were identified as an important pool of stem cells that contribute to the maintenance of suture patency at postnatal stages, as well as injury repair, in adult mice. Notably, postnatal ablation of *Gli1*-expressing cells in mice led to coronal synostosis [51]. Subsequent studies identified other markers of the sutural stem cell population, such as *Axin2, Prrx1*, and *Ctsk*, and demonstrated the capacity of these cells to regenerate bone [52].

Another process during suture genesis is ***lineage commitment***, which is a process controlled by transcription factors, such as zinc finger protein of the cerebellum 1 (ZIC1), engrailed 1 (EN1), MSX2, and TWIST1, that are expressed in the supraorbital regulatory center. Studies in mouse models revealed that *En1* is important for both the position and maintenance of a lineage boundary between the mesoderm and the neural crest-derived cells along the developing coronal suture (**Figure 4**). Additionally, a study proposed that *En1* acts upstream of *Msx2* and *Twist1* and prevents the early *Fgfr2*-mediated differentiation of sutural osteoprogenitor cells [5]. Mutations affecting the transcription factor *ZIC1* in humans lead to syndromic coronal synostosis with learning disabilities. Studies in *Xenopus* and murine embryos supported a role for *Zic1* mutations in altered *En1* expression in the supraorbital regulatory center along with disruption of the normal patterning of the coronal suture [53]. Additionally, studies in mice showed that *Twist1* is expressed early in mesenchymal sutural cells, especially at the osteogenic bone fronts, and negatively regulates *runt-related transcription factor 2* (*Runx2*), a master regulator of osteogenic differentiation, thereby maintaining sutural patency [54]. Loss-of-function mutations in *TWIST1* lead to a classical CS syndrome (Saethre–Chotzen), and the presence of multiple copies of *RUNX2* accompanied by their likely overexpression are associated with CS, whereas loss-of-function mutations lead to cleidocranial dysplasia with delayed closure of fontanels and sutures [25, 26, 55-57]. Moreover, TWIST1 interacts synergistically with TCF12 to form a heterodimer with a likely role in formation of the boundary between the neural crest and cephalic mesoderm, which is possibly associated with limited osteogenic differentiation via inhibition of RUNX2, bone morphogenic protein (BMP), or FGFR signaling. Mutations in *TCF12* lead to reduction of the heterodimer activity and are an important cause of CS (mainly coronal) [43]. *Msx2* is important for maintaining the osteoprogenitor population in the developing calvaria, as

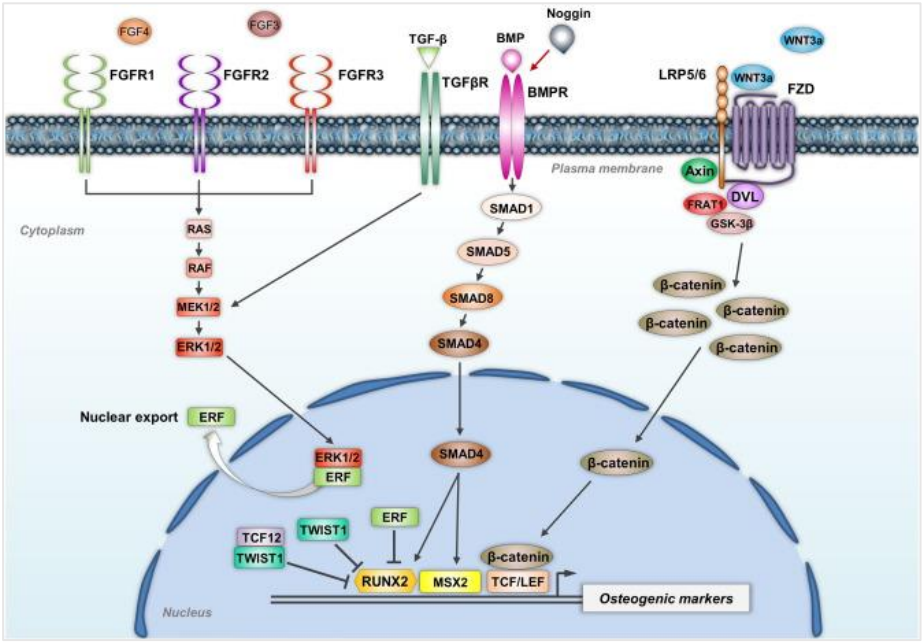

Figure 5. Signaling pathways implicated in suture development. Illustration from Katsianou, Adamopoulos [6] (https://doi.org/10.1016/j.bbacli.2016.04.006) reproduced under Creative Commons user license (https://creativecommons.org/licenses/by-nc-nd/4.0/).

Msx2-deficient mice present defects of skull ossification, with loss-of-function mutations in humans resulting in similar cranial bone defects (parietal foramina). Notably, *MSX2* was the first candidate gene for CS with gain-of-function mutations detected in families with Boston-type CS[38, 58]. Other signaling pathways, such as those involving wingless (WNT), BMPs, and FGFs, are implicated in the commitment to osteogenic fate (**Figure 5**). For example, FGF2 upregulates *Twist1* expression, whereas BMP2 upregulates *Msx2* expression. [50, 59].

The third process is represented by ***boundary formation and integrity of the cell lineages*** within the suture and occurs in close coordination with the previously described processes. This is of particular importance for the coronal suture, which presents dual embryonal origin. Cells from the neural crest and cephalic mesoderm migrate to form the frontal and parietal bones, respectively. Even in the presence of a neural crest/mesodermal boundary, studies in mice show that a population of multipotent cells in the mid-sutural mesenchyme contribute to the growth of both parietal and frontal bones. Depending on their position within the suture, the cells can be recruited into the developing bones if located near the osteogenic fronts, whereas mid-sutural cells remain

undifferentiated. The maintenance of this population of undifferentiated cells is essential for suture integrity. *Twist1*-deficient mice present a disorganized boundary with ectopic presence of neural crest-derived cells in the parietal bone and an expanded expression of *Msx2*. . Additionally, a previous study showed that altered EFNA4 (ephrin-related receptor tyrosine kinase ligand 4) expression affects boundary formation, with *EFNA4* mutations identified in patients with non-syndromic coronal synostosis, whereas *EFNB1* (ephrin B1) mutations lead to craniofrontonasal syndrome with coronal synostosis [60]. Furthermore, JAGGED/NOTCH signaling also plays a role in boundary formation, as loss-of-function mutations in *JAG1* lead to Alagille syndrome, which often presents with CS, and *Jag1* inactivation in mesodermal cells of the coronal suture results in CS [51, 60-63].

The fourth process is represented by the ***osteogenic proliferation and differentiation*** of cells, which are essential for growth of the skull bones following expansion of the underlying brain. Hedgehog (HH) signaling is important to craniofacial development, and Indian HH (IHH), which plays a role in chondrocyte proliferation and differentiation, is also an osteogenic marker in cranial bone formation. CNVs involving noncoding elements at the *IHH* locus are associated with CS and syndactyly in humans, and *Ihh*-deficient mice show reduced levels of Bmp2/4 along with defective bone formation and delayed mineralization with wide sutures corresponding to the undifferentiated mesenchyme. Therefore, IHH secreted at the osteogenic fronts is thought to play a role in maintaining the recruitment of osteoprogenitor cells from the suture along with *IHH* overexpression leading to CS [51, 64, 65]. Additionally, RUNX2 is a driver of osteogenic differentiation in both endochondral and intramembranous ossification and its relation to TWIST1 has been previously commented.

Another key factor for skull growth involves FGF signaling (**Figure 6**). Gain-of-function mutations in *FGFR* genes represent the most frequent cause of SCS (Apert, Crouzon, Pfeiffer, and Muenke syndromes) and have been critical to expanding the understanding of craniofacial development. Elevated levels of FGF stimulate osteogenic differentiation at the bone fronts, whereas low levels are required at the sutural level to maintain patency. Notably, the dura mater is also a source of FGFs (FGF2 in particular), with other growth factors [BMP4 and transforming growth factor-beta (TGF-β)] initiating stimulating effects during early suture genesis until establishment of the intrinsic sutural production[4]. In murine models, *Fgfr2* is expressed by proliferating osteoprogenitor cells, and its downregulation marks the onset of osteogenic differentiation along with upregulation of *Fgfr1*. *Fgfr3* is expressed at low

levels in bony regions, whereas elevated expression is observed in the cartilaginous plate underlying the lower part of the coronal suture [66].

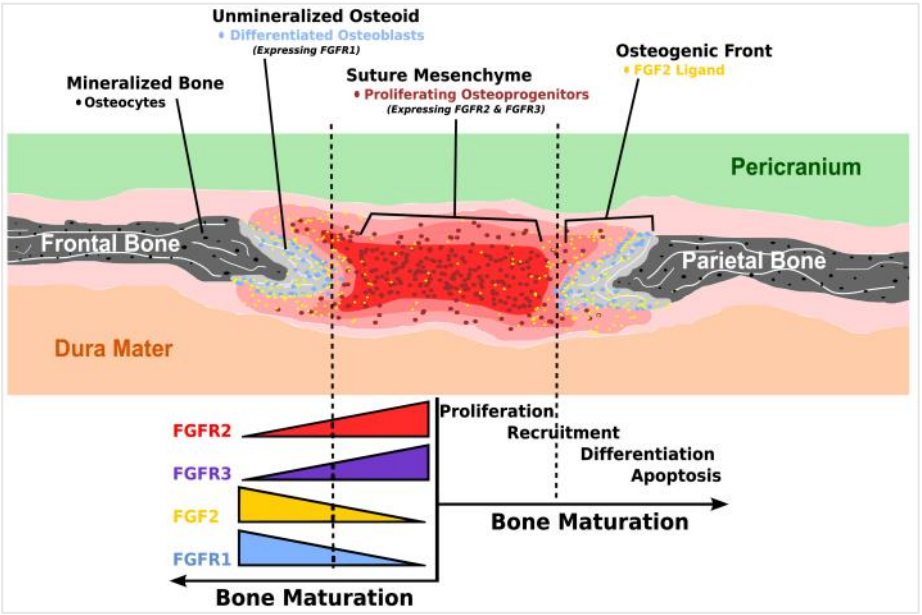

Figure 6. FGF-signaling during coronal suture development. Under low levels of FGF2*, the undifferentiated osteoprogenitor cells from the sutural mesenchyme proliferate expressing* FGFR2 *and* FGFR3*. Elevated levels of* FGF2 *at the bone fronts lead to the recruitment of osteoprogenitor cells into osteoblasts with increased expression of* FGFR1 *and deposition of osteoid matrix along the osteogenic fronts. Illustration from Teven, Farina [4] (https://doi.org/10.1016/j.gendis.2014.09.005) reproduced with permission from the authors and the Editor of Genes and Diseases.*

The downstream pathways activated by FGF signaling include those involving RAS/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK), which affect cellular proliferation and differentiation, phosphoinositide 3-kinase/Akt (PI3/AKT) involved in cell survival, polarity, and fate determination, and phospholipase C-γ involved in cell morphology, migration, and adhesion [4]. Interestingly, missense variants leading to equivalent amino acid substitutions in the extracellular immunoglobulin-like linker regions (IgII–IgIII) of FGFR1/2/3 (p.Pro252Arg in FGFR1, p.Pro253Arg in FGFR2, and p.Pro250Arg in FGFR3) cause phenotypically overlapping but distinct syndromes that affect the coronal suture (Pfeiffer, Apert, and Muenke syndromes, respectively) (**Figure 7**) [50]. A likely explanation for the similarities is the increased binding affinity and broadened specificity of the mutant receptors for FGF10. Furthermore, other mutations in these genes, especially *FGFR2*, lead to different patterns of synostosis not

always involving the coronal suture, such as in Crouzon syndrome. Moreover, specific mutations in *FGFR1/2/3* also affect endochondral ossification that leads to skeletal dysplasias, such as osteoglophonic dysplasia and achondroplasia. These differences may be explained by the impact of different mutations on the secondary and tertiary structures of the proteins, as well as the ligand affinity of different splicing isoforms [67, 68]. Notably, *de novo* recurrent mutations affecting specific positions (mutational hot spots) in proteins from the FGF and RAS signaling pathways reportedly occur at higher frequency in the male germinal line and give selective advantage to the mutant cells. This phenomenon is referred to as 'selfish spermatogonial selection' and is observed in the Apert, Noonan, and achondroplasia syndromes [69].

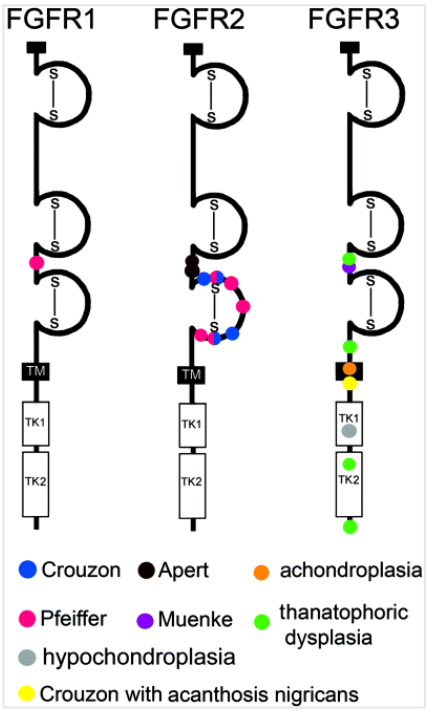

Figure 7. Location of several recurrent mutations in FGFRs with the associated disorders. The receptors contain three Ig-like domains sustained by disulphide bonds (s–s); TM - transmembrane domain; TK1,2 - tyrosine kinase domain. Illustration from Morriss-Kay and Wilkie [2] (https://doi.org/10.1111/j.1469-7580.2005.00475.x) reproduced with permission from John Wiley and Sons (License no: 5401600580220).

Although several mutant mice harboring the corresponding human mutations were created, it was difficult to distinguish a timeline for the disturbed developmental processes. Additionally, these models presented synostosis of the facial bones rather than CS. One of the most frequently used mouse models is the Apert-*Fgfr2*$^{Ser252Trp/+}$ mutant, in which acceleration of proliferation and differentiation at the osteogenic fronts were observed following decreases in the undifferentiated sutural stem cell population [70]. Mutations in genes encoding downstream effectors of signaling pathways, such as *ERF*, lead to syndromic forms of CS often accompanied by delayed postnatal multiple-suture synostosis [42, 71]. Furthermore, mutations affecting other genes in the RAS/MAPK/ERK pathway (i.e., *PTPN11*, *KRAS*, *BRAF*, and *SHOC2*) have been observed in patients with a Noonan-like phenotype and CS, suggesting a complex

interrelationship between these signaling pathways[72]. Notably, downstream signal attenuation is mediated by the sprouty RTK signaling antagonist (SPRY) family of proteins (MAPK phosphatases) activated by FGF signaling, suggesting an autoinhibition mechanism [4]. The crossing of mutant mice with a conditional allele of *Spry1* and *Twist1*$^{+/-}$, respectively, prevented the occurrence of CS in the offspring, indicating that TWIST1 acts upstream of FGF signaling, and that reduced osteogenic differentiation can compensate for the loss of boundary integrity caused by TWIST1-deficiency [50]. Interestingly, loss-of-function variants in *SPRY1* and *SPRY4* were reported in patients with midline NCS [73]. Ras-associated binding protein 23 (RAB23) is another negative upstream regulator of both FGF and HH−GLI1 signaling and in which bi-allelic mutations cause Carpenter syndrome [Online Mendelian Inheritance in Man (OMIM)#201000] accompanied by multiple-suture CS and polydactyly [74].

Another factor implicated in osteogenic differentiation involves the dysregulated metabolism of retinoic acid, which exerts teratogenic effects on the limb and craniofacial skeleton. Bi-allelic mutations in *POR* and *cytochrome P450 26B1* (*CYP26B1*) lead to syndromic forms of CS, such as Antley–Bixler with genital anomalies and disordered steroidogenesis (OMIM#201750) and CS with radio-humeral fusions and other skeletal and craniofacial anomalies (OMIM#614416), respectively. Additionally, *CYP26B1*-related syndromes can present with mineralization defects of the skull. Studies in zebrafish and mice show that defective degradation of retinoic acid is associated with impaired osteoblast–osteocyte transitioning [75]. Interestingly, results from a recent study performing transcriptome analysis of isolated suture-derived mesenchymal stem and progenitor cells from *Erf*-deficient mice showed an increase in CYP26B1 levels accompanied by subsequent increases in retinoic acid catabolism and defective osteogenic differentiation. The authors concluded that ERF might represent a link between the FGF and retinoic acid signaling pathways [76].

Mutations in genes encoding ciliary proteins lead to a large group of disorders referred to as ‘ciliopathies’ accompanied by pleiotropic phenotypes, including CS (e.g., Bardet–Biedl syndrome, Joubert syndrome, and cranioectodermal dysplasias). The HH and WNT pathways use ciliary proteins for signal transduction, and cilia are essential for cellular migration and mechanotransduction (**Figure 8**) [1]. Additionally, mechanical stress reportedly influences the physiology of sutures by upregulating genes that control osteogenic differentiation [77]. Other studies demonstrated the role of the primary cilium on the apical surface of osteogenic precursors in the transduction of mechanic stimuli and activation of the osteogenic cascade [78-

80]. Based on these results, a role for the primary cilium in the etiopathogenesis of NCS has been considered [1].

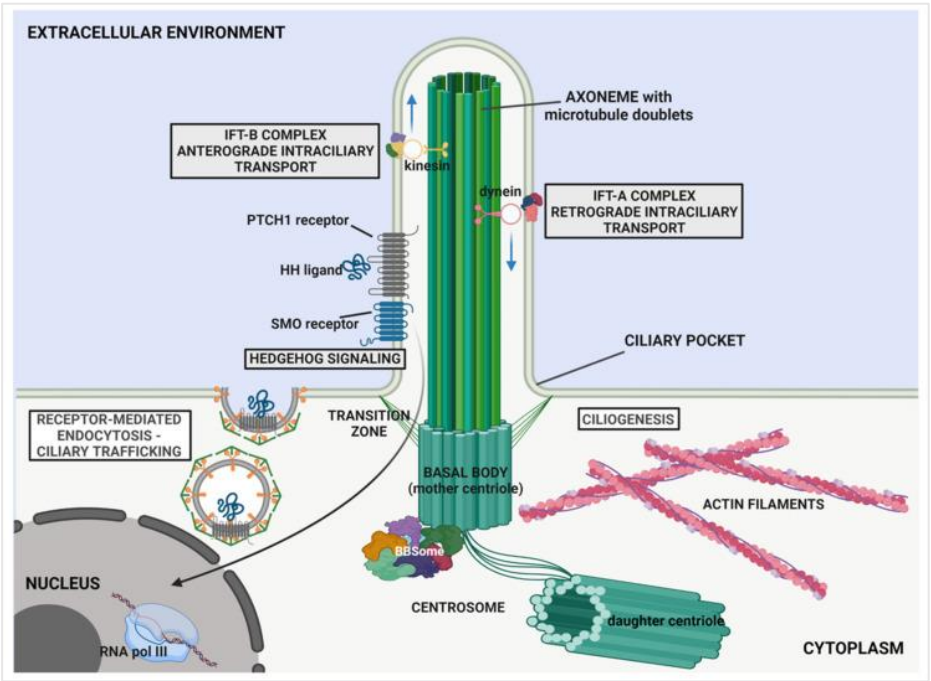

Figure 8. Representation of the primary ciliary structure and its relation to the HH-pathway. Proteins and other cellular structures are transported by intraflagellar transport (IFT) and kinesin-motor proteins. Illustration from Tiberio, Parolini [1] (https://doi.org/10.3390/genes12071073) reproduced under Creative Commons user license (https://creativecommons.org/licenses/by/4.0).

Finally, the ***balance between osteogenesis and resorption*** is an important factor for ***homeostasis*** of the mature suture [50]. Resorption is mediated by osteoclasts, and bi-allelic mutations in *IL-11-receptor alpha* (*IL11RA)* lead to a Crouzon-like phenotype often accompanied by pansynostosis, as well as delayed eruption of secondary dentition [81-83]. Previous studies of mouse osteoblasts and bone marrow cells suggest a central role for IL-11 (a GP130-coupled cytokine) in osteoclast development, as well as a potential effect on both bone-forming and -resorbing cells expressing IL11-RA [84]. Furthermore, mutations in *IL6 cytokine family signal transducer* (*IL6ST/GP130*) have been reported in patients with immunodeficiency and CS, supporting the role of the cytokine IL-11 in bone remodeling and resorption [85-88].

Genes encoding the aforementioned key regulators of craniofacial development (e.g., *FGFRs*, *TWIST1*, and *TCF12*) associated with syndromes

in which CS is a constant feature may be considered core genes related to CS, as proposed by Twigg and Wilkie [50]. Another group is represented by genes associated with syndromes in which CS occurs secondary to the perturbation of other primary processes and in which its occurrence varies. Examples include disorders of bone metabolism and homeostasis (e.g., hypophosphatasia, osteopetrosis, osteosclerosis, and mucopolysaccharidosis). Additionally, a particular subgroup in this category includes genes that encode proteins involved in cell division and DNA repair and in which mutations lead to poor brain growth with severe forms of microcephaly with sometimes secondary CS (e.g., Seckel syndrome spectrum and other disorders with primary microcephaly). A third emerging and heterogeneous group is represented by genes encoding proteins involved in different developmental pathways, such as transcription factors or chromatin remodelers [e.g., *lysine-specific methyltransferase 2D* (*KMT2D*) and *lysine acetyltransferase 6B(KAT6B)*], in which mutations lead to pleiotropic and variable effects, such as CS. The number of genes associated with SCS is constantly increasing as a result of the rapid development of NGS techniques, which allow rapid and effective screening of the whole genome.

1.4 CRANIOSYNOSTOSIS TYPES – CLINICAL CLASSIFICATION AND ITS LIMITS

From a clinical standpoint, CS can be classified according to the presence or absence of other malformations, dysmorphic features, and/or neurodevelopmental abnormalities in SCS or NCS forms. A strict delineation between SCS and NCS is challenging and depends on factors, such as patient age, the clinical experience of the examiner, and/or the subjective assessment of the craniofacial phenotype. Additionally, a clear distinction between syndromic and nonsyndromic presentations may be difficult as in the case of apparently NCS with neurodevelopmental problems in the absence of increased ICP and independent of the type of surgery, suggesting a primary abnormality of brain growth and development [7, 89-91]. The presence of neurodevelopmental problems without obvious dysmorphic features and/or other morphological anomalies besides CS often results in a classification as NCS. Moreover, mild dysmorphic features make it difficult to distinguish between secondary craniofacial deformations due to CS (e.g., orbital asymmetry and nasal deviations with facial scoliosis or low-set, asymmetric positioning of the ears) and primary features of a syndromic presentation. These aspects may influence the epidemiology of NCS as reflected by the variability in the reported share of SCS (15-30%) [8, 15, 92].

Furthermore, inherited forms of CS may present significant variability in intrafamilial expressivity and even incomplete penetrance of the pathogenic DNA variant along with mildly affected or asymptomatic family members, as observed in Muenke, Saethre–Chotzen, and other syndromes with autosomal dominant inheritance. This can lead to the wrong assumption that a familiar case of CS is nonsyndromic and sporadic (*de novo*) if the parents do not present obvious craniofacial changes [8].

Another classification uses the synostotic pattern: single-suture synostosis (sagittal, metopic, coronal or lambdoid – **Figure 9**) or multiple-suture (multisuture) synostosis involving two or more sutures and including pansynostosis (from the Greek prefix ‘pan’ meaning ‘all’), which represents the early closure of all cranial sutures. Single-suture synostosis represents ~70% of CS cases. Different cranial deformations can be observed depending on which suture is closed: sagittal synostosis leads to scaphocephaly, metopic synostosis leads to trigonocephaly, unilateral coronal (unicoronal) synostosis leads to anterior plagiocephaly, and unilateral lambdoid suture leads to posterior plagiocephaly. Single-suture synostosis is most frequent among NCS

cases, with the highest prevalence of 1 in 5000 live births for sagittal synostosis, followed by metopic synostosis (between 1 in 7000 and 1 in 15,000

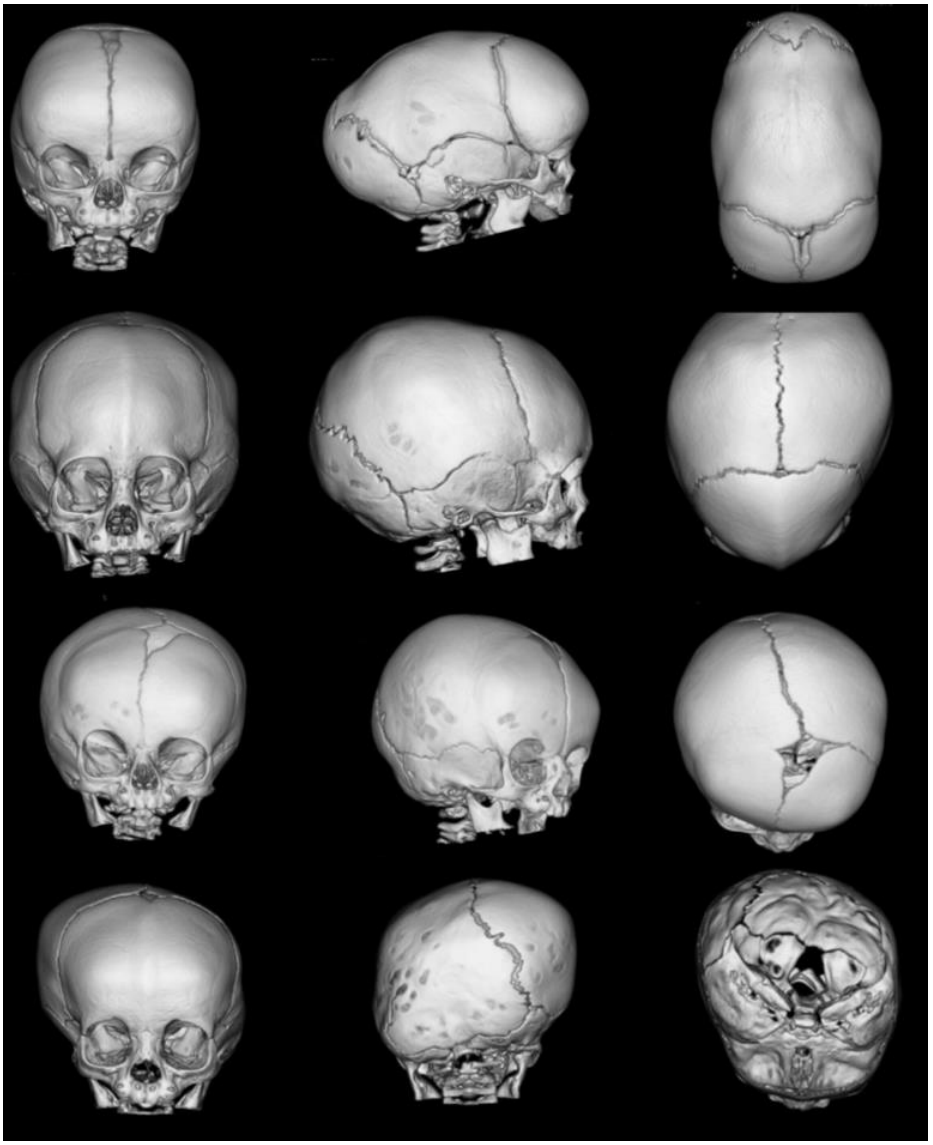

Figure 9. 3D-CT reconstructions of skulls presenting four types of single-suture synostosis from upper to lower panel: sagittal synostosis with scaphocephaly, metopic synostosis with trigonocephaly, right unicoronal synostosis with right anterior plagiocephaly, left lambdoid synostosis with left posterior plagiocephaly. Compiled illustrations reprinted from Blessing and Gallagher [3] (https://doi.org/10.1016/j.coms.2022.02.001) with permission from Elsevier (License no: 5402001140994).

live births), coronal synostosis (1 in 10,000 live births), and lambdoid synostosis (1 in 33,000 live births). A gender imbalance is noted for sagittal and coronal synostosis, with the former being more prevalent in boys and the latter more prevalent in girls [93-95]. Notably, in both familial and sporadic cases of coronal synostosis associated with the *FGFR3* variant p.Pro250Arg (Muenke syndrome, the most frequent genetic cause of CS), females present a more severe phenotype (bicoronal synostosis) than males, suggesting the possibility of modifier genes [96]. Multiple-suture synostosis is frequent among SCS cases with predominant coronal suture involvement, especially bicoronal synostosis in Apert, Saethre–Chotzen, and Muenke syndromes.

In summary, the clinical classification of CS is challenging due to the heterogeneity of the phenotypic presentations in terms of synostotic pattern, severity, and the presence of other symptoms. However, advances in genetic analyses have offered insight into the etiology of CS by explaining both the similarities and differences between the clinically defined diagnoses. In this regard, recurrent mutations in *FGFR2* were identified in Apert syndrome and Beare–Stevenson cutis gyrata syndrome (BSS), both of which present distinct phenotypic features, whereas other specific mutations have been observed in both Pfeiffer and Crouzon phenotypes. Muenke syndrome is the typical example of a molecularly defined diagnosis (recurrent mutation in *FGFR3*) accompanied by significant familial variability with both syndromic and nonsyndromic presentations. However, these aspects also complicate the understanding of CS in regard to genotype–phenotype correlations.

1.4.1 SYNDROMIC CRANIOSYNOSTOSIS

A majority of SCS cases are represented by classical, relatively easily recognized syndromes, such as Apert, Crouzon, Pfeiffer, and Muenke syndromes and craniofrontonasal dysplasia (**Appendix**). These represent at least two-thirds of all syndromic presentations of CS and are caused by mutations in the CS core genes *FGFR2, FGFR3, TWIST1*, and *EFNB1* [15]. The first four syndromes have an autosomal dominant inheritance pattern but may occur sporadically as a result of *de novo* mutations, as in severe forms of Pfeiffer syndrome and most cases of Apert syndrome. Craniofrontonasal syndrome is caused by mutations in *EFNB1* located on the X chromosome and typically presents with coronal synostosis. Paradoxically, females are more affected than males due to a phenomenon referred to as 'cellular interference', which disturbs the process of boundary formation between the neural crest and mesoderm-derived tissues at the coronal sutural level. Affected females present cell subsets with different levels of EFNB1 expression due to random X inactivation [40].

Other less frequent but clinically recognizable CS syndromes include Antley–Bixler, Crouzon syndrome with acanthosis nigricans, BSS, Jackson–Weiss syndrome, Carpenter syndrome, Greig cephalopolysyndactyly syndrome, osteoglophonic dysplasia, Shprintzen–Goldberg syndrome, Loeys–Dietz syndrome, Baller–Gerold syndrome, Opitz trigonocephaly C syndrome, Bohring–Opitz syndrome, and other rare syndromes accompanied by variable occurrence of CS. Clinical and genetic details of clinically recognizable syndromes are summarized in **Appendix**.

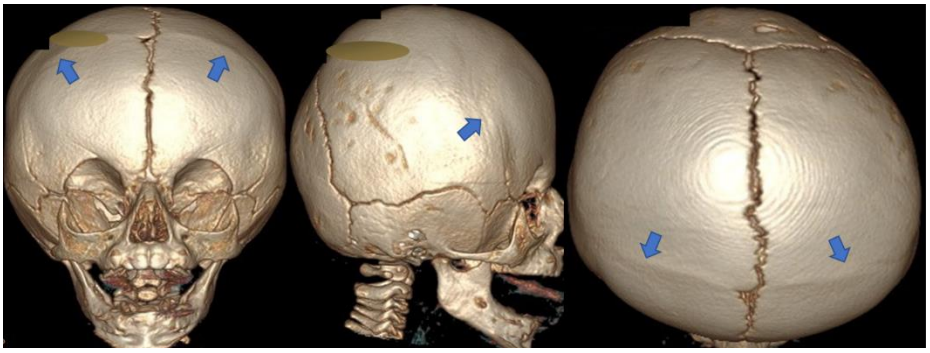

Figure 10. 3D-CT reconstruction of skull with bicoronal synostosis (blue arrows) resulting in brachycephaly, frequently observed in classic CS syndromes.

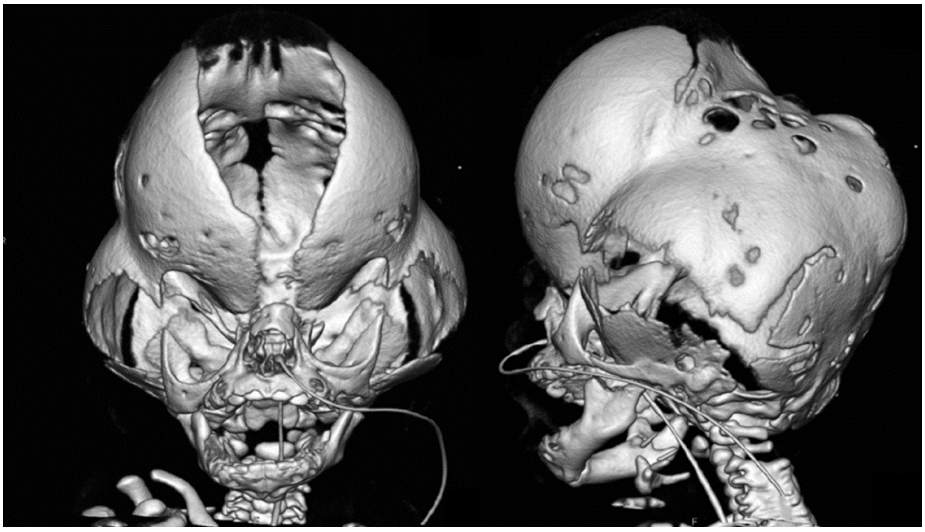

Figure 11. 3D-CT reconstruction of skull with cloverleaf deformation resulting from multiple suture synostosis as observed in Pfeiffer type 2 and Apert syndromes.

The FGF signaling pathway plays a major role in skeletal development, as revealed by the diverse conditions caused by mutations in *FGFR* genes [97]. A typical example is *FGFR2*, mutations of which are associated with a variety of syndromes, including those related to CS. Interestingly, there are genotype–phenotype correlations between specific mutations, such as p.Ser252Trp and p.Pro253Arg with Apert syndrome and p.Ser372Tyr and p.Tyr375Cys with BSS; however, the mutational spectrum is broader in Crouzon and Pfeiffer syndromes. Both Apert and BSS have a distinct phenotype that reveals the roles of different FGFR2 domains and isoforms in cranial and limb patterning during embryonal development. Limb abnormalities are usually not present in Crouzon syndrome in contrast with the severe syndactyly that characterizes Apert syndrome [23]. Notably, insidious postnatal pansynostosis is most common in Crouzon syndrome [98]. Additionally, the p.Trp290Cys mutation is associated with the occurrence of a tracheal cartilaginous sleeve with significant mortality risk in Pfeiffer patients [99].

Mutations in other CS core genes, such as *MSX2*, *TCF12*, and *ERF*, may lead to an unspecific phenotype associated with either NCS or SCS along with a clinical picture which sometimes mimics other syndromes, such as Crouzon or Saethre–Chotzen. Relatively few CS cases caused by *MSX2* mutations have been described after that in the original Boston family with variable forms of craniofacial deformation, including cloverleaf skull [38]. Notably, *ERF*-related CS often occurs postnatally following an insidious progression, with delayed diagnosis at ~3 or ~4 years of age and associated neurodevelopmental

problems [100, 101]. *TCF12*-related CS may present with features of Saethre–Chotzen syndrome, which is expected based on the synergistic interaction of TCF12 with TWIST1 [43].

Chromosomal abnormalities represent up to 15% of the genetically explained cases of CS [15], with several chromosomal changes, including deletions, duplications, and unbalanced translocations, having been described. Many of these involve CS-related genes, such as 7p21.1 deletion (*TWIST1*), 9p22.3 deletion (*FREM1*-related trigonocephaly), and 6p21 duplication (*RUNX2*). Wilkie et al. [8] noted the predominance of midline synostosis (metopic/sagittal) in a cohort of patients with chromosomal abnormalities. Other chromosomal anomalies, such as 11q23-ter deletion (Jacobsen syndrome) and both deletions and duplications at 22q11 often present with CS; however, no strong candidate genes for CS have yet been detected [7].

Moreover, diverse chromosomal imbalances involving CS-related genes or genes with pleiotropic effects, including sutural development (such as transcriptional regulators), must be considered in the differential diagnosis of SCS forms presenting a different phenotype from those of classical CS syndromes.

1.4.2 NONSYNDROMIC CRANIOSYNOSTOSIS

The etiology of NCS is multifactorial and remains elusive in most cases. Although intrauterine environmental factors may contribute to CS occurrence, it is difficult to prove in individual cases. In a 13-year birth cohort of CS patients treated at a single craniofacial unit, only 2.4% of cases revealed a likely secondary cause (extreme prematurity in three cases of sagittal and two cases of metopic synostoses, and maternal valproate in six cases of metopic synostosis) [15]. Fetal constraint is another suggested etiology with increased risk in cases of nulliparity, twining, breech position, and male sex (to explain the higher incidence of sagittal synostosis in boys with larger heads); however, different studies produced conflicting results [102]. Therefore, systematic prospective studies with well-characterized cohorts in terms of pregnancy type and follow-up of maternal and fetal factors could elucidate a possible role for fetal constraint in CS.

In the previous decade, results from exome/genome sequencing analyses suggested a stronger involvement of genetic factors in NCS etiology. Timberlake et al. [73] reported *SMAD family member 6* (*SMAD6*) mutations in 7% of patients with midline synostosis (sagittal/metopic). Importantly, reduced penetrance (inheritance from an unaffected parent) was observed, suggesting an epistatic interaction with a common variant in the vicinity of *BMP2*, which augmented the penetrance of *SMAD6* variants [73]. However, a recent study could not confirm an association between presence of the *BMP2* risk allele and CS occurrence in patients with *SMAD6* mutations [103]. Furthermore, Timberlake et al. [73] found truncating variants in *SPRY1* and *SPRY4*-genes (sprouty RTK signaling antagonists) in patients with sagittal NCS as well as a de novo missense variant in *SMAD-specific E3-ubiquitin protein ligase 1 (SMURF1)*, a binding partner of SMAD6 in a patient with metopic synostosis.

Another study showed that a group of splice isoforms of BBS9 (from autosomal recessive Bardet–Biedl syndrome type 9; also known as *parathyroid hormone-responsive B1*) were overexpressed in fused suture specimens from patients with midline NCS as compared with matched controls [104]. The results suggest a role for BBS9 in the morphology of the primary cilium and the abnormal ossification of the sutural osteogenic niche. Recently, a novel paternally inherited missense variant of *BBS9* was reported in a patient with coronal NCS [105]. Mutations in genes encoding components of the primary cilium cause a large group of disorders (ciliopathies) that present pleiotropic phenotypes, including CS (as observed in cranioectodermal dysplasias). Furthermore, an emerging mechanosensory role was proposed for the primary

cilium in the detection and transduction of osteoinductive signals (including environmental ones) accompanied by activation of the intracellular osteogenic cascade [1, 79].

Interestingly, mutations in *insulin-like growth factor 1 receptor* (*IGF1R*) were detected in patients with single-suture NCS, with transcriptomic studies revealing upregulation of the IGF1 signaling pathway in calvarial osteoblasts from patients [106, 107].

Mutations in CS core genes (*FGFR1, FGFR2, FGFR3, TWIST1, TCF12,* and *EFNB1*) have been described in certain cases of apparent NCS [43, 105, 108, 109]. It is important to consider the difficulties in the clinical assessment of mildly affected individuals and the significant variability in intrafamilial expressivity and incomplete penetrance of CS disorders. From a sutural standpoint, coronal and metopic synostoses appear to have a stronger genetic background, with familial recurrence in up to 10%, whereas a positive family history is found in 2% to 5.7% of sagittal synostosis cases [7, 108, 110, 111].

A recent study identified enrichment in *de novo* variants in several mutation-intolerant genes in children with NCS [112]. Notably, the burden of *de novo* variants correlated with a higher incidence of neurodevelopmental delay and intellectual disability in these children. Many of the reported candidate genes (56) currently have no disease association, but nearly all of the remaining 26 genes (coding for chromatin modifiers, transcriptional regulators, components of the ubiquitin–proteasome system, etc.) are involved in neurological or neurodevelopmental disorders. However, none of the disorder-associated genes [except *fibrillin 1(FBN1)*] has an established role in CS. The authors suggest that the DNA variants are hypomorphic alleles that could result in CS with a concomitant effect on neurodevelopment. These results emphasize the importance of the initial clinical assessment of patients in terms of SCS versus NCS, as well as the follow-up of patients with apparent NCS that could develop primary neurodevelopmental issues regardless of surgical intervention. A recent Swedish study of the neuropsychological outcomes of children operated for NCS revealed average levels of intelligence quotient accompanied by mild attention and vigilance weaknesses of likely no clinical importance and unrelated to the surgical procedure (spring-assisted surgery or pi-plasty) in the sagittal and metopic synostosis groups [113].

The rapid development of high-throughput sequencing methods during the previous decade enabled the discovery of new candidate genes for both SCS and NCS; however, interpretation of the clinical significance of different DNA variants, especially in the case of NCS, remains challenging when considering

the possible low-penetrance and modifier (epistatic) effects of co-segregating alleles supporting a polygenic background.

2 AIMS

The scope of this thesis was to investigate the genetic factors underlying CS occurrence in a retrospective cohort of patients undergoing surgery at the largest reference center in Sweden.

Owing to the retrospective and observational nature of this study, the specific aims crystallized into four studies:

I. To study the prevalence of CS and the genotype–phenotype correlation in patients with Kabuki syndrome and patients with 10q22.3q23 microdeletions starting from a unique case of a patient with sporadic occurrence of both *KMT2D* mutation and 10q22.3q23 microdeletion.

II. To study the prevalence and spectrum of genetic alterations in patients with CS involving the coronal suture, whether isolated or in combination with other suture synostoses (excluding pansynostosis).

III. To study the prevalence and spectrum of genetic alterations in patients with syndromic forms of sagittal and pansynostosis.

IV. To analyze patients without a detected causal genetic alteration at targeted screening by using WES and WGS methods to allow exome/genome-wide analyses.

3 METHODOLOGICAL APPROACHES

3.1 CLINICAL ASPECTS

Initial recruitment phase (until 2016) for targeted NGS screening

Patients and relatives were retrieved from the Gothenburg Craniofacial Registry and covered the majority of cases addressed for craniofacial assessment at the largest reference center of craniofacial surgery in Sweden up to 2016.

Retrospective phenotyping

A thorough review of patient medical records, including photos and 3D-CT skull reconstructions, was performed by a clinical geneticist (AT) in order to characterize the cohort in terms of sutural pattern and presence of associated symptoms (SCS vs. NCS).

SCS was suspected in patients presenting one or several of the following features:

1. Significant changes in craniofacial morphology affecting the eyes (e.g., shallow orbits with proptosis, hypertelorism), maxilla (e.g., maxillary hypoplasia with relative prognathism), nasal pyramid (e.g., small, beaked), and ears (e.g., dysplastic helix, low-set ears).
2. Neurodevelopmental abnormalities (e.g., intellectual disability, seizure disorder).
3. Other anomalies (e.g., cleft palate, heart defect).

The particular case of a boy presenting SCS reminiscent of Kabuki syndrome, in which a de novo 10q22.3q23.1 microdeletion was detected by clinical screening, led to an in-depth analysis, including a follow-up clinical examination, as well as a retrospective review of previously reported similar cases.

Phenotypic subgrouping according to sutural pattern

We then explored the distribution of genetic alterations in different synostotic patterns in our cohort. Following retrospective phenotyping, the patients were divided into two groups according to the main sutural involvement:

1. Coronal synostosis isolated or combined with other suture synostoses (Coronal Plus) (excluding pansynostosis).
2. Sagittal synostosis isolated or combined with other suture synostoses (Sagittal Plus) and pansynostosis.

This separation was based on the complexity of the embryofetal development of the coronal suture, which is the most frequently affected suture in SCS [5, 8]. Additionally, pansynostosis often presents an insidious postnatal course that can correspond to a distinct genetic background.

Collection of DNA material

Blood samples (in EDTA tubes) were collected in connection with surgical interventions from patients who provided informed consent to participate in the study. The samples were stored in Biobank 446 (according to the Swedish regulations) at the Department of Clinical Pathology and Cytology, Sahlgrenska University Hospital. This biobank is governed by the Swedish Biobanks in Medical Care Act (SFS 2002:297).

Secondary recruitment phase (2016–2020) for WGS/WES analyses

Additional patients that agreed to participate in the study were recruited according to clinical laboratory records showing negative outcomes following clinical diagnostic work-ups between 2016 and 2020 using *in silico* CS-related panels on exome/genome data. Retrospective phenotyping was performed, as previously described, although no phenotypic subgrouping by sutural pattern was performed, given that this cohort included patients with negative outcomes at initial screening either in a research or clinical setting using targeted methods.

Collection of DNA material

We used blood collected in EDTA tubes and stored in the biobank at the Department of Clinical Genetics and Genomics, Sahlgrenska University Hospital. This biobank is governed by the Swedish Biobanks in Medical Care Act (SFS 2002:297).

In the majority of cases, parental samples were unavailable due to a lack of systematic collection. In certain cases, samples could be obtained *a posteriori* for segregation analysis in cases of potentially relevant variants of uncertain clinical significance (VUS).

3.2 TECHNICAL ASPECTS

Targeted NGS sequencing

A targeted NGS panel including 63 CS-related genes was designed in collaboration with the SciLife Laboratory (Solna, Sweden). The panel included the core genes associated with CS (*FGFR1, FGFR2, FGFR3, TWIST1, EFNB1, RAB23, MSX2, POR,* and *TCF12*) and other genes infrequently associated with CS. Gene selection was performed using the OMIM (https://www.omim.org/) and PubMed (https://pubmed.ncbi.nlm.nih.gov/) databases. Genes involved in endocrine/metabolic disorders, where CS occurs as a secondary event (e.g., *ALPL* in hypophosphatasia), were not included. CNV analysis was not performed.

WES/WGS analyses

WES/WGS was performed on patients without detected causal variants at the initial targeted screening. Due to the lack of parental samples, single exome/genome analysis was performed in the majority of cases, except for two: 1) trio analysis in a patient with sporadic SCS and 2) quadro analysis including two affected siblings and their mother with sagittal NCS and the unaffected father. Human Phenotype Ontology (HPO)-driven software (Moon diploid, Invitae®) was used to filter variants from exome/genome-wide data according to a list of genetic variants associated with the given phenotypic terms. Different software was used for *in silico* analysis of a larger CS-related gene panel that included 133 genes (Alissa Interpret, Agilent® Technologies). Another round of HPO-driven analysis limited to CS-related terms was subsequently performed on the exome/genome-wide data. We performed complementary analyses, such as microarray (SNP-microarray), MLPA, cDNA analysis to evaluate splicing effects, targeted variant analysis to determine either parental segregation or confirm NGS results, as needed.

The case of SCS with Kabuki-like features

The patient was screened using both the targeted NGS panel (63 genes) and a clinical commercial panel to confirm the results. The latter included seven genes for Kabuki and phenotype-overlapping disorders and 34 CS-related genes.

3.3 VARIANT INTERPRETATION AND STATISTICAL ANALYSIS

Variant interpretation

Variant assessment was performed manually, with the variants classified according to the American College of Medical Genetics criteria [114], as follows:

- Pathogenic (class 5)
- Likely pathogenic (class 4)
- VUS (class 3)
- Likely benign (class 2)
- Benign (class 1)

The criteria account for whether a variant was previously reported in the literature, the presence or absence in databases of normal and pathologic variations, the frequency in the normal population, conservation among species, and *in silico* predictions of effects at the protein level. Additionally, genotype–phenotype correlations were determined. This is an essential and individualized step in variant interpretation which is complicated by the heterogenous clinical presentations and complex genetic background of different disorders. Moreover, the variant assessment is limited when detailed phenotypic information about the parents and parental samples for segregation analysis are not available.

Statistical analysis

Because this represents a descriptive, observational study, advanced statistical analyses were unnecessary. Statistical processing of phenotypic and genotypic data included grouping the patients into different categories depending on the type of synostosis, SCS versus NCS, presence or absence of causal variants in the respective groups, and the distribution of genetic variants in the different groups. This was followed by calculation of the percentage of patients in each category. Data were processed and graphic representations created using functions in Excel (Microsoft Corp., Redmond, WA, USA). The Genome Aggregation Database (https://gnomad.broadinstitute.org/) was used to obtain the variant frequencies in the control populations.

3.4 OUTCOME OF THE CLINICAL AND LABORATORY WORK-UP

Targeted NGS screening

DNA was isolated and met the quality criteria in 144 individuals from 135 unrelated families retrieved from the Gothenburg Craniofacial Registry. Five samples were from relatives with similar or milder phenotypes, whereas another four samples were from unaffected parents. We retained 126 individuals (122 patients + 4 unaffected parents) from 117 unrelated families after exclusion of 18 patients due to incomplete phenotypic information (15 cases) and insufficient DNA allowing verification of low-quality raw-data results after the first screening (3 cases).

WES/WGS analyses

Of the 40 patients with negative results at the first screening, 37 were included and three excluded due to insufficient DNA. Twenty-two additional patients with negative results at clinical screening from 2016 to 2020 were retrieved from the laboratory records of the Department of Clinical Genetics and Genomics, Sahlgrenska University Hospital. Eventually, 62 individuals (59 patients and 3 unaffected parents) from 57 unrelated families were retained. A summary of the patient materials used, and methods applied for each study is shown in **Table 1**.

Table 1. Summary of the patient materials and methods used in the different studies.

Study No.	**Clinical approach and no. of patients**	**Technical approach**
I	Clinical assessment Literature review One patient with combined unicoronal and sagittal synostosis and Kabuki-like phenotype with *de novo* 10q22.3-q23.1 microdeletion	Targeted NGS panel (63 CS-related genes) - research Commercial targeted NGS panel (41 genes, Kabuki- and CS-related) for the confirmation of initial results Karyotype and SNP-microarray analyses performed as part of the clinical diagnostic work-up
II	Retrospective phenotyping 100 patients (95 unrelated families) with coronal synostosis	Targeted NGS panel (63 CS-related genes)
III	Retrospective phenotyping 18 unrelated patients with sagittal and pansynostosis SCS (Three patients with sagittal NCS and negative outcome were reported in Study IV)	Targeted NGS panel (63 CS-related genes)
IV	Retrospective phenotyping 59 patients (57 unrelated families): • 37 cases from targeted NGS screening • 22 additional cases	WGS/WES Complementary karyotyping, SNP-microarray, MLPA, cDNA analysis, and parental segregation were performed if samples were available

3.5 ETHICAL ASPECTS

The primary activities of this study included collection of medical records and analysis of inheritable factors of disease concerning the genetic integrity of each respective patient. The study was reviewed and approved by The Regional Ethical Review Board in Gothenburg (No. 303-15), and informed consent to participate in the study was obtained from all patients or their parents. In the case of children (usually infants) and disabled individuals, the legal guardian provided consent. The results are coded and can be traced only by the medical doctors and clinical scientists involved in the study. The data were handled according to the Ethical Review Act (2003:460), the General Data Protection Regulation, GDPR (2016/679), and the Swedish Biobank in Medical Care Act (2002:297). Written consent for the publication of patient images included in Study I was obtained from the parents/guardians. The studies did not involve any medical risks for the patients.

The use of detection methods, such as WES/WGS, provides information about all known genes and, therefore, introduce a risk of unintentional detection of a previously known mutation in a gene not linked to CS but signaling a predisposition for another disease later in life and without the present display of symptoms at study inclusion (e.g., mutations associated with hereditary forms of cancer). This could represent a benefit for the individual and the family, because they could be included in prevention and control programs related to the disease. However, some individuals may not want to know in advance whether they are at risk for a certain disease. This raises the question of reporting or not reporting possible incidental findings. This can be handled by providing genetic counselling to the families. However, these situations are rare owing to both the rarity of such diseases in the population and because no active search for these variants is performed, as the variant-filtering process focuses on phenotypically relevant genes. Furthermore, it is possible to limit the detection of secondary findings by adapting the search options of the analytical tools exclusively toward genes related to the phenotype (i.e., use of HPO-driven software). Awareness of the possibility of identifying variants in genes previously unrelated to the phenotype is required; therefore, careful assessment of the limitations of the search tools is necessary to ensure that potentially relevant information for the study is not eliminated. When similar methods are applied in clinical diagnostic settings, patients are informed by the clinicians regarding the implications of these tests in terms of incidental findings. However, this is not the case for research studies, where data analysis is handled differently. For example, the visualization of an identified variant

in a gene linked to hereditary cancer does not provide information concerning its possible pathogenicity, as it could be a benign variant or a technical artefact [such variants need to be carefully assessed in order to be classified as pathogenic, likely pathogenic, VUS, or benign (polymorphic)]. In some cases, even a complementary analysis is necessary in order to determine whether the finding is a true variant or an artefact. In such cases, these steps can be regarded as an active search for secondary findings, although not the purpose of the present study and not covered by the patient's informed consent.

In summary, the methodology used in this particular research setting, which was focused on phenotypically relevant genes for CS, does not pose the same problems concerning secondary findings as an analysis performed in a clinical setting. The variant review boxes in the filtration pipelines used in Study IV (WES/WGS analyses) were adapted to the research purpose of the present analysis.

4 MAIN RESULTS

Study No.	Results
I	*De novo* truncating variant in *KMT2D* [c.1033_1036delCTCT,p.(Leu345Valfs*56)] confirming the predicted clinical diagnosis • The patient represents a rare case of *de novo* occurrence of two distinct disease-causing genetic alterations at the chromosome and gene levels, respectively Literature review • Eight cases of Kabuki syndrome with CS • 13 cases of overlapping deletions with 10q22.3–q23.1 (including *NRG3*), and descriptions of facial asymmetry and skull deformations (plagiocephaly, dolichocephaly, and frontal bossing), suggesting underlying CS
II	**Clinical findings** • 78% were SCS; gender ratio, 1:1 **Genetic findings** Causal findings: • 80% SCS, 14% NCS • *FGFR2, TWIST1, FGFR3, TCF12, EFNB1, POR,* and *FGFR1*, with five novel variants in *TWIST1, TCF12,* and *EFNB1* • *FGFR2* p.Ser252Trp was the most prevalent mutation (Apert syndrome) • Two siblings with a *TWIST1* deletion and one patient with an unbalanced translocation (5pter-p15.3 trisomy + 9pter-p24.2 deletion) diagnosed in a clinical testing

	• Co-segregating VUSs with possible modifier effects in four patients with causal variants. • Additional VUSs in seven patients without causal variants. • Novel VUSs in *SPECC1L, IGF1R,* and *CYP26B1*
III	**Clinical findings** • Multiple synostosis in 89% of cases (9 pansynostoses and 7 Sagittal Plus) • Gender ratio, 3.5:1 (14 males vs. 4 females) • Prevalent phenotypic presentation in the pansynostosis subgroup was Crouzon or Crouzon-like **Genetic findings** Causal findings: • 83% with higher proportion among pansynostosis cases • One new case of *SKI*-related CS (Sagittal Plus; Shprintzen–Goldberg syndrome) • Three new cases of *IL11RA*-related pansynostosis with a Crouzon-like phenotype. Review of 17 previously reported cases revealed the predominance of pansynostosis with insidious postnatal progression and risk of increased ICP. • One patient harboring a *PHEX* mutation, and another showing a chromosomal aberration (19.2-Mbp inverted telomeric duplication of 15q25.2q26.3 including *IGF1R*) were diagnosed during clinical testing. • Co-segregating VUSs in two patients with causal variants. • Two additional VUSs: one novel in *RUNX2* and one in *AXIN2* in a patient without causal variants, and presenting with a Carpenter-like phenotype and combined metopic and sagittal synostoses
IV	**Clinical findings** • SCS in 51% of the families, with a predominance of multisuture synostosis in 62% (bicoronal in 17%), unicoronal in 28%, and sagittal in 10%

	• NCS with following distribution of the synostotic patterns: unicoronal in 57%, multisuture in 30% (with 7% bicoronal), and sagittal in 13%. • Gender distribution, 1:1.2 (27 males and 32 females) **Genetic findings** Causal findings: • 38% of SCS with novel variants in the genes: *FGFR2* (one rare case with an Alu insertion), *TWIST1*, *TCF12*, *KIAA0586*, *YY1*, *HDAC9*, *FOXP1*, and *NSD2*. • New case of recurrent truncating *KAT6A* mutation (Arboleda–Tham syndrome). • Two *de novo* chromosomal anomalies: 1) a complex structural rearrangement involving duplication of 2p25.2 (including *SOX11)* and 2q22 deletion; and 2) a duplication of 22q13.1-q13.2 (including *EP300*). • The contribution of WGS/WES to the diagnostic yield by group of patients initially screened in research and clinic, respectively: 16% of the patients analyzed with targeted NGS-panel in research and 25% of the patients analyzed with in-silico panel in the clinic. • Potentially relevant VUSs (of which 34 are novel) in 87% of the remaining families without detected causal variants. • Potentially relevant VUSs in NCS patients [e.g., *ERF*, *CHD7* (familial sagittal synostosis), *SPRY1*, and enrichment of variants in genes involved in ciliopathies and the TGF-β pathway]. • Overall: 115 genetic variants (11 causal, 95 potentially relevant, 9 unlikely to contribute to the phenotype) were detected. 47 variants (41%) were detected by using the 133-genes *in-silico* panel whereof nine (8%) exclusively by this method, 102 variants (89%) were detected by HPO-driven filtration whereof 57 (50%) only by one filtering software (Moon), and 6 variants (5.2%) (all causal) were detected by CNV-analysis (the *FGFR2*-Alu-insertion was exclusively detected by CNV-analysis; the *HDAC9*-deletion was flagged by the panel analysis and confirmed by CNV-analysis; the duplications 2p25.2 and 22q13, and the *NSD2*-deletion were flagged by HPO-filtration and confirmed by CNV-analysis).

5 DISCUSSION

This thesis presents an evaluation of the genetic background of CS in a cohort of patients addressed for intervention at the largest reference center for craniofacial surgery in Sweden. The nature of the study was retrospective and descriptive. Initial targeted NGS, followed by exome/genome-wide analyses of cases with negative results demonstrated a high diagnostic yield in SCS. The use of state-of-the-art analytical methods, such as WES/WGS, combined with HPO-driven variant filtration enabled the molecular diagnosis of rare forms of CS. The results contribute to a better understanding of genotype–phenotype correlations in CS by confirming previous observations and identifying new associations.

5.1 CO-SEGREGATING VARIANTS – A CHALLENGE TO PHENOTYPIC INTERPRETATION

The development of massively parallel sequencing methods has revealed a complex and surprising reality. The clinical diagnostic journey of the patient described in Study I is illustrative in this sense. The patient had undergone routine clinical testing on a syndromic indication comprising SNP-microarray as the first-tier analysis, which revealed the 3.2-Mbp 10q22.3q23.1 microdeletion, with subsequent parental testing revealing *de novo* occurrence. The case was considered solved from a genetic standpoint, and the author was the clinical geneticist who met the patient and his parents for genetic counselling 4 years prior to performing the targeted NGS screening. At that time, the child was 6 months of age, and the clinical picture was reminiscent of Kabuki syndrome. The few reported patients with 10q22.3q23.1 deletions and in which craniofacial deformities were described suggested that CS was part of the phenotypic spectrum of the microdeletion syndrome. Because several genes in the deletion encode proteins with unknown function, one could suspect the presence of a candidate gene for Kabuki syndrome that appears to have a heterogeneous genetic background with at least two known associated genes (*KMT2D* and *KDM6A*). In up to 20% to 30% of patients with typical features of Kabuki syndrome, the genetic cause remains unknown [115]. If the patient had not been included in this study, the *KMT2D* causal variant might have been missed.

Identification of the loss-of-function mutation in *KMT2D* was not surprising in this case considering the clinical suspicion of Kabuki syndrome; however, the sporadic occurrence of two causal genetic changes with potential combined phenotypic effects was surprising. Notably, application of NGS has changed the classical view of a monogenic cause for a syndromic presentation and revealed the complexity of these rare but not exceptional situations. In this sense, the significance of CS is difficult to interpret in this context. The aggregation of several congenital abnormalities in an individual is usually considered to have a common etiology, which is often the case, but the occurrence of two genetic changes may have a combined effect difficult to distinguish. Additionally, the known variable expressivity of autosomal dominant conditions may be a confounder. The previous reports of CS in Kabuki patients support a causal role for *KMT2D* in sutural development. As noted in the manuscript for Study IV, mutations in genes encoding proteins involved in the epigenetic machinery (in this case, a lysine-specific methyltransferase) have pleiotropic effects at the genomic level by changing

the chromatin structure, thereby altering the expression of several genes, including those involved in osteogenic processes. Kabuki syndrome is a typical example of a 'chromatinopathy', as these disorders have been named. Simultaneously, a role for the 10q22.3q23.1 microdeletion in CS cannot be excluded, given the previously reported cases of cranial deformation suggesting an underdiagnosed synostosis.

This case highlights the importance of a thorough clinical examination of patients *prior* to genetic testing and reinforces the critical role of a 'clinical eye'. This may be determinant in cases with a typical phenotype suggesting a specific syndrome but a negative outcome at genetic screening. A closer look at the raw data or re-analysis of specific genes with other methods may detect an initially missed variant or a particular genomic structural change possibly involving noncoding regulatory regions not routinely analyzed and that lead to alterations in gene expression. This was the case for the patient with typical Apert syndrome (P_3 in Table 1 of Paper IV), where closer examination of the raw genomic data following negative results during routine analysis of *FGFR2* identified a rare Alu insertion.

The growing use of WGS in the clinic enables early detection of co-segregating chromosomal abnormalities with sequence alterations, which is important for disease prognosis and appropriate genetic counselling of the family. Another relevant example reported in Paper IV (Table 1 and Discussion) concerns a patient with sagittal SCS and a *de novo* duplication of 22q13.1–q13.2. No previous associations with CS were found, and analysis confirmed a previously known paternally inherited VUS in *MSX2* detected at initial screening using a targeted NGS panel. The sagittal synostosis was related to the possibly low penetrant *MSX2* variant; however, absence of known craniofacial deformations in the father's family and the molecular aspects of the variant (synonymous without a predicted splice effect) suggested no phenotypic impact.

Furthermore, we have detected a multitude of co-segregating variants in both patients with and without detected causal variants. These variants are located in CS core genes, genes involved in osteogenic pathways, and/or genes associated with syndromic presentations accompanied by variable occurrence of CS. Although their clinical significance is uncertain (VUSs), a possible modulator effect cannot be excluded, given the extreme heterogeneity of CS in terms of both clinical presentation and genetic background.

5.2 IMPORTANCE AND LIMITATIONS OF PHENOTYPE CLASSIFICATION – ARE WE MOVING TOWARD A GENE-RELATED CLASSIFICATION?

As previously discussed, a clear distinction between NSC and SCS may be difficult due to for example the variable expressivity of the disorders both intrafamilial and between unrelated cases, the young age of a patient without a fully developed phenotype, and the examiner's experience and subjectivity. A retrospective analysis makes it even more difficult.

Additionally, classification by sutural pattern has its limitations, especially in SCS, where multiple suture synostoses with complex deformation are frequent, and the precise extent of the synostosis pattern may be difficult to determine. This was the case of a patient with an uncertain synostosis pattern, an atypical Shprintzen–Goldberg-like phenotype, and who was not included in one of the phenotypic subgroupings (coronal vs. sagittal/pansynostosis) and had negative results at the initial targeted NGS screening. Closer examination of the medical records, including 3D-CT images, revealed that the patient had combined bicoronal synostosis, metopic and possibly sagittal synostosis, and a recurrent mutation detected in *KAT6A* by WGS analysis (Table 1 in Paper IV) and compatible with the recently described Arboleda–Tham syndrome (a 'chromatinopathy'). However, the subgrouping of patients by main sutural pattern in Studies II and III was useful, and the data confirmed previously known phenotype–genotype correlations, such as predominance of coronal synostosis in Apert and Crouzon syndromes. Notably, this also suggested the prevalence of insidious postnatal pansynostosis in patients with mutations in *IL11RA*, suggesting an important role of IL11RA in maintaining the balance between osteogenesis and resorption in all sutures.

In Study IV, the sutural pattern was dominated by coronal involvement in both SCS and NCS (Figures 1A, 2, and 3B in Paper IV). This is explained by the biased clinical recruitment of cases that underwent surgical intervention at our center, which specializes in complex and rare forms of CS. Interestingly, the highest diagnostic yield by sutural pattern was recorded for sagittal SCS, although the number of cases was significantly lower as compared with coronal SCS (Figure 3B). Furthermore, potentially relevant variants have been detected in a large proportion of patients with coronal NCS, as well as in the minor group of sagittal NCS (Figure 3C), which supports the role of genetic factors

in NCS, especially at the coronal sutural level with its complex biogenesis. Notably, we identified an enrichment of variants in genes associated with 'ciliopathies' (disorders of the primary cilium), with potential implications for the mechanotransduction of signals at the sutural level, as well as in genes associated with TGF-β signaling and with reported roles in sutural genesis and patency [1, 116].

At the same time, it was noted that recurrent mutations may be associated with different phenotypic presentations and synostotic patterns with the typical example of *FGFR2*-variants associated with both Crouzon and Pfeiffer phenotypes. A variation in the synostosis pattern with predominance of a certain suture (in particular coronal) is observed for the most frequently mutated CS core-genes (*FGFR2, TWIST1, ERF, TCF12*). Furthermore, CS occurs sporadically in a multitude of syndromes which emphasizes the complexity of the mechanisms controlling suture patency both during embryofetal development and postnatally.

Twigg and Wilkie [50] noted the extreme heterogeneity of CS in terms of both clinical presentation and genetic background and proposed a genetic–pathophysiologic framework for classifying CS. Depending on how frequently mutations in certain genes are associated with CS, two broad groups were distinguished: the "core genes", in which >50% of the mutations are associated with CS, and the remaining genes, in which only a minority of mutations caused CS.

5.3 TARGETED ENRICHMENT NGS PANELS VERSUS *IN SILICO* ANALYSIS OF GENOMIC/EXOMIC DATA – WHAT TO CHOOSE IN CLINICAL PRACTICE?

There are no established criteria for the selection of CS patients for whom genetic screening is indicated; however, in general, a syndromic presentation prompts further analysis. Different studies propose diagnostic algorithms depending on the presence or absence of additional findings, which suggest a syndromic presentation, suture closure pattern, and/or positive family history [117, 118]. Judging from the prevalence of classic syndromic presentations caused by mutations in the core genes, the idea of using a limited targeted panel (e.g., *FGFR1/2/3, TWIST1, TCF12, EFNB1, ERF, POR,* and *IL11RA*) as a first-tier approach in clinical testing seems to be preferred. Additionally, the financial aspect needs to be taken into account, as the use of larger panels that include genes rarely associated with CS may seem cost ineffective [119]. However, knowing the aforementioned limits of the clinical classification and the multitude of syndromes in which CS has been described, a larger panel will have a higher diagnostic yield in SCS, as demonstrated by previous studies and the present results [120]. Furthermore, knowledge concerning genetic causes of CS is in constant and rapid flux along with the regular discovery of new candidate genes, thereby introducing challenging situations for clinical laboratories required to adapt their diagnostic pipeline [121]. The recurring question remains how many genes to include in a panel that must be validated in terms of effective coverage of the analyzed regions. The validation steps are time-consuming, and the panel needs to be updated according to cases of newly discovered genes. Moreover, the diagnostic pipeline should include complementary methods (e.g., MLPA) for genes with reported CNVs (deletions/duplications), such as *TWIST1* and *EFNB1*.

Given the results from previous studies and the present findings, WGS seems preferable in cases of SCS [122, 123]. *In silico* panels can be adapted to the clinical presentation using a stepwise approach, which allows the use of a panel limited to the CS core genes first in case of classic syndromic presentations but with the possibility of expanding the analysis to a larger panel in case of negative results. For nonspecific syndromic presentations, screening could start with a larger panel with the possibility for genome-wide analysis in the next step. In case of apparent NCS, the presence of coronal or multisuture engagement can be used as an indicator for testing. Additionally, the aggregation of several cases of CS and/or craniofacial deformation in a family

is a strong indicator of a genetic background in NCS. A suggestion of a diagnostic workflow for CS is shown in **Figure 12**.

Introduction of WGS/WES in the diagnostic routine for CS should take several aspects into account. The amount of data generated by each analysis requires access to a large storage facility and an effective bioinformatics pipeline for processing raw data. The present findings demonstrated the effectiveness of HPO-driven variant filtration for WGS/WES analyses in detecting CS-related variants in genes not known to be associated with CS and/or in recently discovered candidate genes, which were not included in the *in silico* panel. However, HPO-driven filtration may lead to an overflow of variants that are difficult to interpret in nonspecific cases or those presenting a poorly characterized phenotype related to the syndromic presentations, as well as in cases of NCS. Therefore, this application requires a robust workflow for variant interpretation, during which allele frequencies and other molecular aspects are considered. Furthermore, use of parental samples in a trio analysis is preferable, as it enables rapid and effective variant interpretation via direct access to information about inheritance. Notably, the use of complementary microarray should be considered in cases of SCS and if the WGS platform is not adapted for reliable detection of CNVs.

In conclusion, WGS is an effective tool for detecting pathogenic variants in SCS and can potentially increase the diagnostic yield in cases of NCS with a suggested polygenic background. Nevertheless, improvements to the algorithms employed for variant calling and interpretation are needed along with a closer collaboration between researchers and clinicians in order to maximize the potential of genome sequencing in clinical routines [124].

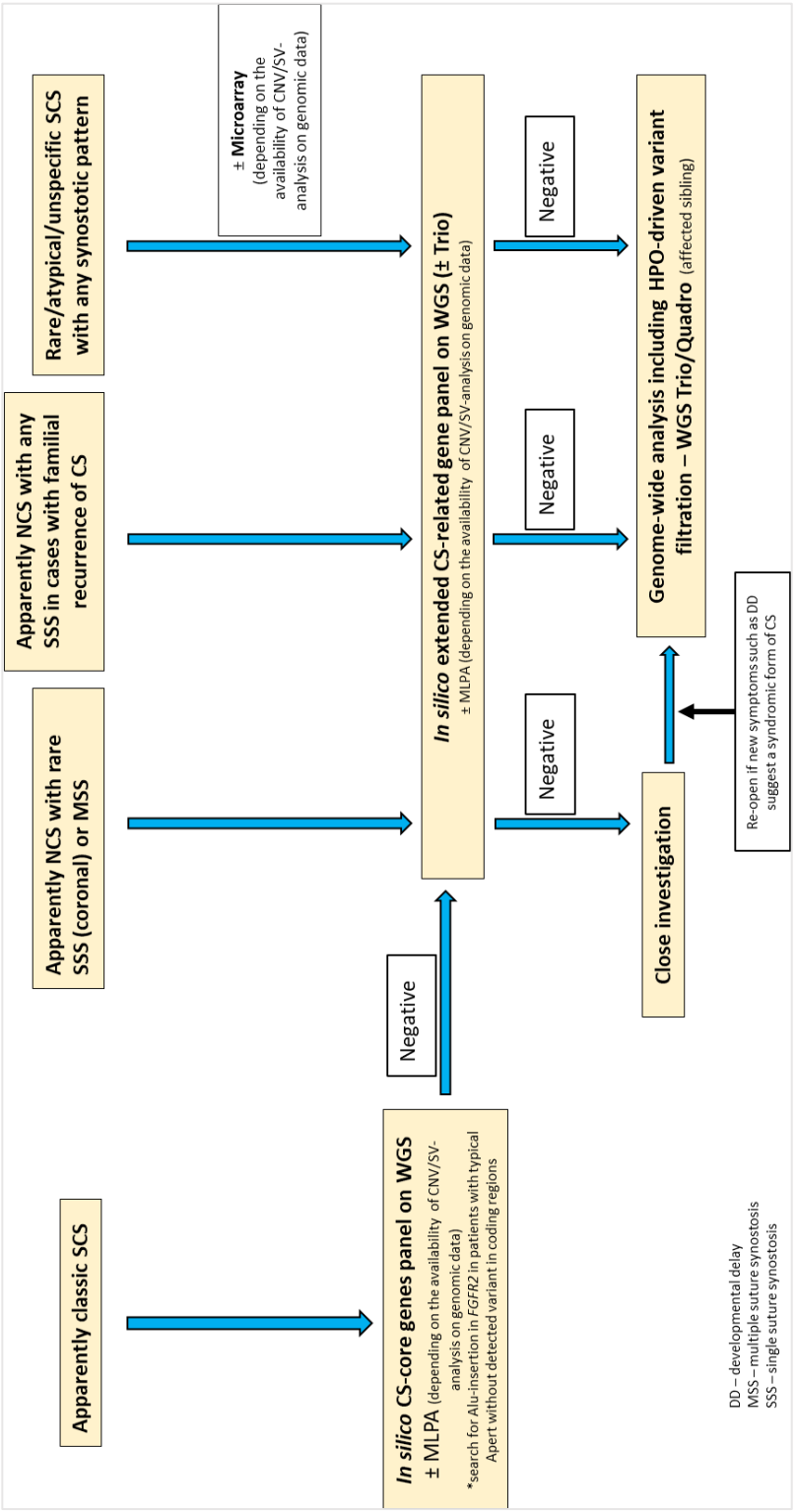

Figure 12. Suggested diagnostic workflow in CS.

5.4 LIMITATIONS IN THE STUDY DESIGN AND METHODOLOGICAL APPROACHES

The retrospective nature of this study made phenotype assessment challenging. The patients up to 2016 were initially retrieved from the Gothenburg Craniofacial Registry without an in-depth phenotyping and a focus on cases with coronal involvement and more complicated synostoses that could suggest a genetic cause. An initial targeted screening using an NGS panel of 63 CS-related genes was performed on the available DNA samples, which fulfilled the quality criteria, prior to detailed phenotype characterization. This 'blind' approach from genotype to phenotype had its pros and cons. It offered the advantage of a rapid preliminary analysis with the identification of previously known pathogenic variants in CS core genes accompanied by confirmation of a genotype–phenotype correlation, which in the majority of cases corresponded to the clinically suspected diagnosis. Moreover, this approach allowed identification of the case of a boy with SCS, in whom a *de novo* microdeletion detected by clinical testing was considered diagnostic. The patient's phenotype strongly suggested another monogenic cause that was confirmed by targeted sequencing in research setting and resulted in closer analysis and follow-up of the patient (Study I). However, the *a posteriori* phenotype characterization of the cohort in parallel with interpretation of the genetic results in other cases with more complex phenotypes (not classical CS syndromes) was time-consuming and prevented access to an overall assessment of the structure of the cohort in terms of SCS versus NCS forms, as well as sutural pattern, prior to the analysis. This led to the exclusion of patients with incomplete phenotypic information after screening. However, a strength of our study was the detailed phenotype description of the patients despite the limited information in certain cases, as well as the uniform assessment performed by one examiner using the same criteria for the phenotype subgrouping.

Another drawback was the lack of parental samples in the majority of cases, which hampered the interpretation of VUSs, especially in Study IV. This was partially addressed in certain cases, for which parental samples could be obtained *a posteriori*, although only for targeted variant analysis.

The capacity of the analytical pipeline to detect certain types of genetic variants was also a limitation. This was particularly evident in cases of CNVs detected by WGS and for which the lack of an effective bioinformatics filter and method for annotating variants led to time-consuming manual interpretation.

Additionally, we were unable to perform CNV analysis of the sequencing data from targeted NGS and whole-exome analyses, and complementary microarray analysis was not possible for all SCS cases.

6 CONCLUSIONS

Study I

CS may be an underdiagnosed feature of both Kabuki syndrome and the phenotypes associated with 10q22.3q23.1 deletions.

A thorough phenotypic assessment prior to genetic screening increases both the quality of variant interpretation of genotype–phenotype correlations and the diagnostic yield in cases of stepwise analytic approaches.

The use of WGS enables simultaneous detection of causal genetic variants of different types, such as chromosomal anomalies and point mutations, in the same individual.

Study II

Coronal synostosis has a strong genetic background. Causal variants were detected in 80% of SCS cases and 14% of NCS cases. Additionally, novel variants were observed in *TWIST1, TCF12,* and *EFNB1*.

Targeted NGS screening is an effective method for detecting causal variants in patients with coronal synostosis, and the content of the gene panel can be adapted to include both classical and rare syndromic presentations.

Study III

Causal variants were detected in 83% of cases and in higher proportion among pansynostosis cases. Despite significant phenotype variability associated with genetic variants in the same gene, certain synostosis patterns are recurrently associated with mutations in specific genes. Our findings identified *IL11RA* as an emerging core gene for autosomal recessive pansynostosis. Additionally, mutations in *SKI* show a tendency to affect the sagittal suture.

Broad genetic screening of patients with suspected syndromal forms of CS with multiple suture involvement is important for early diagnosis and follow-up of lesser-known forms of CS with insidious postnatal progression and risk of increased ICP, such as in *IL11RA*-related pansynostosis.

Study IV

Causal variants were detected in 38% of SCS cases, including novel variants in *FGFR2* (Alu insertion), *TWIST1*, *TCF12*, *KIAA0586*, *YY1*, *HDAC9*, *FOXP1*, and *NSD2*, as well as two structural genomic rearrangements involving chromosomes 2 and 22. Moreover, potentially relevant variants (of which 34 were novel) were detected in 87% of the remaining unrelated SCS and NCS cases without detected causal variants.

WGS is an effective tool for the diagnosis of rare forms of SCS and returns an increased diagnostic yield as compared with targeted analyses. Additionally, use of HPO-driven variant-filtration software improved detection of phenotype-relevant variants in both SCS and NCS cases.

7 FUTURE PERSPECTIVES

The results of this study highlight the importance of a craniofacial team in the diagnosis, management, and follow-up of patients with CS. Together with plastic surgeons and other healthcare specialists (e.g., pediatricians and psychologists), clinical geneticists play an important role in the initial assessment of patient phenotype, which ideally should be performed prior to genetic analysis in order to provide a reliable interpretation of the results. Moreover, early interaction between the patient's parents and family with the clinical geneticist allows them the opportunity to discuss different aspects of CS in terms of recurrence risk, heredity, associated symptoms, and genetic diagnostic methods. Furthermore, in addition to periodic postoperative controls, patients would benefit from a long-term follow-up of psycho-motor development and other phenotypic aspects. This is of particular importance for patients with apparent NCS and who may develop new symptoms, as well as those with undiagnosed SCS after initial genetic screening and who could benefit from reassessment of the phenotype and renewed investigation. An etiologic diagnosis has prognostic value and allows appropriate genetic counseling.

WGS has the capacity to become a unique diagnostic tool for CS, given the adaptability of the method to phenotypic presentation using *in silico* gene panels prior to genome-wide analysis.

Among the most relevant topics in the field of CS research is acquisition of an understanding of the mechanism associated with normal suture development. Studying the genetics of CS patients using high-throughput sequencing methods, such as WGS, enables the discovery of new candidate genes involved in the development of the cranial sutures. Functional studies (both *in vitro* and in animal models) are needed in order to better understand the role of these genes in normal development. Furthermore, larger genetic studies using WGS and transcriptome analyses are necessary to determine the extent of the role of genetic factors in NCS. A better understanding of the genetic factors and genotype–phenotype correlations in CS will improve prognostic capacity and, in certain cases, even influence the choice of surgical intervention [125, 126].

Finally, expanding the knowledge of CS-related etiopathogenetic factors will hopefully enable the development of non-surgical precision therapies for the prevention of early suture closure. Different pharmacological and gene-oriented therapies have been tested on animal models as potential noninvasive strategies for preventing synostosis and suture regeneration in SCS [127-130].

In such cases, it is interesting to note previous observations of neosuture formation in the osteotomy lines of operated, fused sutures that might suggest the repopulation of these zones with activated mesenchymal stem cells from the initial sutural niche and essential for the maintenance of suture patency [131]. Furthermore, a recent study on a mouse model of Saethre–Chotzen syndrome demonstrated that *Gli1*-expressing mesenchymal stem cells combined with modified methacrylate gelatin can support coronal suture regeneration[132].

ACKNOWLEDGEMENTS

And here I am, after this fascinating educational and scientific journey that originated more than twenty years ago in my preclinical years at the faculty of medicine in Bucharest when I had a growing interest in studying genetics and developmental biology. I was fascinated by how such detailed information was coded in the DNA with only four molecules, but of course, things were not so simple as they seemed…

This journey has been shaped by the kind contribution of several people whom I met during my study years and career as a physician, and for whom I would like to express my gratitude.

First, I would like to thank **all the patients** who participated in this study as well as the patients whom I met during my clinical practice and contributed to my formation as a physician.

Professor Lars Kölby, my main supervisor and mentor, you have led my journey through this "research forest" in the most elegant and constructive way! We come from different medical specialties – the "surgeon" and the "geneticist" – but this has never been a hinder, but an advantage. I have learned a lot from our discussions which helped me perceive different aspects of the intricated pathology I was studying. Also, I felt welcomed in your team! Thank you for your always appreciative and confident comments about my work, and for your patience and availability! And last, but not least, your humor which neutralized all your disciple's possible worries arisen along the way! As a previous of your PhD students said, I am ready for another thesis under your supervision!

To my co-supervisors and mentors (AR and GS):

Anna Rohlin, my inspiring colleague and talented researcher, I am glad that you accepted to be my co-supervisor! Your expertise and experience have been so valuable during this complicated journey! Thank you for your help with the analyses and all your time spent to fix the bugs and other problems at the lab. Also, thank you for your careful reading and input about the manuscripts. I appreciated your useful comments about the analytical methods pointing out important aspects of this complex work of interpretation. Thank you for your support along the way and your always encouraging words!

Professor Göran Stenman – Thank you for your always thorough and constructive feedback about my works as well as the encouraging comments! Also, I appreciated your careful and elegant language revisions which refined the manuscripts and improved my English skills. Thank you for your always prompt reply and availability when we needed your opinion about the works, and for your help with laboratory matters.

Lovisa Lovmar, chef of the department of Clinical Genetics – Thank you for your significant work to improve our resources and opportunities for research, as well as for encouraging me to pursue the PhD way! And thank you for your careful readings and input about the manuscripts!

To our colleagues at the labs:

At the Clinical Genetics lab: I would like to thank **Gunilla Kanter Smoler** and **Lena Samuelsson** who helped with the interpretation of complementary analyses including tricky raw-data check-ups. Also, a special thanks to **Ywonne Andrén** and **Josephine Wernersson** from the NGS group, as well as **Eva Portinsson** and **Karin Pettersson** from the sequencing group who performed the bench lab work which generated the analysis data. I would also like to thank **Catarina Darnfors** and **Malin Berggren** from the microarray team for their help with analyses and support, and **Torbjörn Olausson** for the introduction to the diagnostic work-up of craniosynostosis.

At the Cancer research center: **André Fehr** – Thank you for your always prompt and effective assistance with different complementary analyses, and your quick reply when we ran out of DNA. **Mattias Andersson** – Thank you for your careful readings of the manuscripts and useful input.

Also, I would like to thank the craniofacial team: **Madiha Bhatti-Søfteland** for your friendly support and interest in my work, as well as useful discussions, **Peter Tarnow** and **Giovanni Maltese** for your valuable patient descriptions and discussions about the work, **Anna Paganini** for your assistance with the Craniofacial Register and your friendly support along the way, and **Marizela Kljajić** for the interesting and amusing discussions, and your friendly support. Also, a special thanks to the **administrative staff at the Plastic surgery department** who assisted with different organizational matters.

Anna Öfverholm – Thank you for introducing me to Professor Lars Kölby allowing the opportunity to start this research project.

And to **all my colleagues at the department of Clinical Genetics in Gothenburg** – Thank you for your collegial support and pleasant work environment during all these years. A special thanks to my colleagues from **the syndrome team** and to our **genetic counsellors** as well as to my **clinical scientist colleagues,** and **all the managers and staff** at the lab. I also thank **our colleagues from the sample-registration** as well as **our colleagues from the secretariat** for their effective assistance with the processing of research samples and administrative matters.

A special thanks to our previous senior colleagues **Eva Holmberg**, **Mihailo Vujic**, and **Margarita Stefanova**, my internship supervisor, who guided my steps in syndrome diagnostic when I started my career in Gothenburg. I also thank **my colleagues from other specialties** for their collaboration and collegial support along the way.

To my colleagues geneticists in the country – Thank you for welcoming me in the Swedish genetics' society. I have enjoyed our collaboration and your friendly support under all these years. A special thanks to **Professor Magnus Nordenskjöld** who was the first Swedish geneticist I had contact with when I was doing my master's degree in France, and who advised me about how to pursue a career in Sweden.

A special thanks to **the managers, academic, and administrative staff at the Institute of Biomedicine at the university of Gothenburg** who assisted with different matters during my studies and cultivated a welcoming and rewarding research environment. Also, I would like to thank **Anette Nilsson** at the Sahlgrenska Academy's office and **Annelie Grimm** from the printing center for their kind assistance with the cover layout and final formatting of the framework.

To my previous supervisors and mentors:

Professor Henri Plauchu and his colleagues at the department of Medical Genetics in Lyon who guided my first steps in clinical genetics when I was a resident in pediatrics at Edouard Herriot hospital. Thank you for supporting me in pursuing a career as a clinical geneticist and for all the good teaching which has been a strong foundation in my career!

Professor Stanislas Lyonnet at the department of Medical Genetics at the Necker hospital in Paris – Thank you for encouraging me to pursue an education in clinical genetics and register for the Master in Genetics.

Professor Alain Verloes and his colleagues at the department of Medical Genetics at Robert-Debré hospital in Paris where I did my "inter-chu" (inter-regional semester) and acquired solid knowledge in the complicated field of syndromology. Thank you for your teaching and guiding! And a special thanks to **Laurence Perrin** who introduced me to Irina Giurgea, my later supervisor in master research.

Associate professor Irina Giurgea, my supervisor in the master program in Paris who guided my first steps in research and was an inspiring mentor. Thank you for all the good teaching and your support along the way! I recall our discussions about the reasons of doing research, why would someone do a PhD. Now, when I look back, I think that I have chosen the right way. Thank you for your encouragements and your friendship!

I also would like to thank **Professor Michel Goossens and his colleagues and staff** at the department of Biochemistry and Molecular Genetics at the Henri Mondor hospital in Créteil where I have done my bench lab work for the master research program and learned about the diagnostic methods in genetics.

I am also grateful for the solid medical knowledge that I received at the faculty of Medicine Carol Davila in Bucharest. And I would like to thank our late **Professor in physiology Radu Cârmaciu** who once told us at a lecture: "To become a good medical researcher, first you have to be a good clinician."

And last, but not least:

To my *mother* – Thank you for your support, encouragements, and patience on this long journey full of surprises and ups and downs where you always have been close and never doubted me! Mulțumesc pentru tot!

To my *father* – I would have liked to hear your opinion about my first book, not sure if others will follow, but as you used to say – capul sus (head up)!

To my *friends* – Thank you for being there, some of you in different countries, but never forgetting our friendship.

This work has been supported by grants from the ALF-agreement between the Swedish government and the country councils (Swedish Research Council), the Gothenburg Medical Society, and Ågrenska Foundation

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APPENDIX

Summary of best-known and clinically recognizable CS-related syndromes (sources: Muenke et al.[20], Twigg & Wilkie[50], https://www.ncbi.nlm.nih.gov/omim, GeneReviews®)

Syndrome (OMIM#)	**Clinical features**	**Genetics, type of DNA-variants, effects on protein function**	**Inheritance**
Apert (Acrocephalo-syndactyly type 1) (#101200)	Coronal/multisuture synostosis with turribrachycephaly, delayed closure of fontanel, large midline calvarial defect with agenesis of the sagittal suture, jugular foraminal stenosis Megalencephaly Increased risk of hydrocephalus along with skull closure Hearing loss, atresia of external auditory canals DD, ID (variable) Hypertelorism, proptosis, divergent strabismus, short nose, choanal stenosis/atresia, trapezoidal mouth, highly arched palate with median groove because of palatal swellings, cleft palate, bifid uvula, malocclusion, crowding teeth, midface hypoplasia with relative mandibular prognathism Symmetric osseus and/or cutaneous syndactyly of hands and feet ('mitten hands') + other skeletal anomalies (e.g., vertebral fusions, short humerus, radio humeral synostosis) Visceral anomalies of tracheal cartilage, cardiovascular, gastrointestinal, genitourinary	*Gain-of-function FGFR2*-hot-spot variants between IgII and IgIII-like regions: p.Ser252Trp, p.Pro253Arg, other rare variants (e.g. Alu-insertions)	AD (de novo occurrence in most cases, paternal age effect)
Muenke (# 602849)	Coronal synostosis (most often), some patients do not present CS, brachycephaly, macrocephaly, temporal	*Gain-of-function* *FGFR3* – hot-spot p.Pro250Arg (between	AD (both inherited and de novo occurrence with

	bulging, mild midface hypoplasia, hypertelorism, ptosis Sensorineural hearing loss Brachydactyly with broad thimble-like middle phalanges and cone epiphysis, broad halluces, calcaneocuboidal fusion DD, ID (variable)	IgII and IgIII-like regions)	paternal age effect) Highly variable phenotype Incomplete penetrance Females more affected than males
Crouzon (#123500)	Coronal synostosis (most often), sagittal, multisuture Similar, but less severe craniofacial features than in Apert sd. DD, ID (occasional) Conducting hearing loss Abnormal segmentation of tracheal cartilage and cervical vertebrae C2-C3 No limb malformations	*Gain-of-function* *FGFR2* (>50 DNA-variants) – most often located on the IgIII-like domain; same mutations have been associated to a Pfeiffer phenotype	AD (both inherited and de novo occurrence with paternal age effect)
Crouzon syndrome with acanthosis nigricans (# 612247)	Similar craniofacial features as in Crouzon sd. Acanthosis nigricans (neck, axilla, groin, periorbital region, perioral region) Hyperpigmentation, melanocytic nevi, warty acanthomas, papillomatosis, odontogenic tumors	*Gain-of.-function* *FGFR3*– p.Ala391Glu (transmembrane domain)	AD (de novo)
Pfeiffer type 1 **(# 101600)**	Coronal synostosis, midface hypoplasia Normal development (most often) Broad thumbs and great toes, brachydactyly, variable skin syndactyly	*Gain-of- function* *FGFR1* – between IgII and IgIII-like linker regions (most often) *FGFR2* (minority)	AD (both inherited and de novo occurrence)

	Vertebral + other skeletal anomalies (e.g., short humerus, elbow ankylosis) Visceral anomalies		
Pfeiffer type 2 **(# 101600)**	Cloverleaf skull (coronal, sagittal and lambdoid synostosis) Severe ocular proptosis Broad thumbs and great toes, elbow ankylosis Increased risk of DD, ID Early demise	*Gain-of-function* *FGFR2*-cluster in IgIII-like domain, but also in the transmembrane and split kinase domain	AD (de novo)
Pfeiffer type 3 **(# 101600)**	Similar to type 2, multisuture without cloverleaf skull, short anterior cranial fossa		
Jackson-Weiss syndrome (#123150)	Coronal synostosis, acrocephaly, midface hypoplasia Medially deviated broad great toes, short and broad metatarsals, fusion of tarsal and metatarsal bones, cutaneous syndactyly of second and third toes	*Gain-of-function* *FGFR2*– p.Ala344Gly (IgIIIc-domain) – original family	AD (inherited with variable expressivity)
Beare-Stevenson cutis gyrata (#123790)	Multisuture synostosis, cloverleaf skull, low-set ears with preauricular skin-furrows Cutis verticis gyrata, cutaneous and mucosal skin tags, acanthosis nigricans, furrowed palms and soles Anogenital anomalies DD, ID	*Gain-of-function* *FGFR2*– p.Tyr375Cys, p.Ser372Cys, deletions altering the splicing of isoform *FGFR2c* with illegitimate expression of isoform *FGFR2b*	AD (de novo)
Osteoglophonic dysplasia (#166250)	Multisuture (often coronal), cloverleaf skull, frontal bossing, hypertelorism with proptosis, midface hypoplasia with relative mandibular prognathism, short	*Gain-of-function* *FGFR1*-IgIII, transmembrane and kinase domains	Autosomal dominant (often de novo)

	nose with anteverted nares, unerupted teeth Upper airways obstruction with respiratory distress Rhizomelic dwarfism with non-ossifying bone lesions with 'hollow-out' aspect of the metaphyses, fractures, pseudoarthrosis, short and broad metacarpals, metatarsals and phalanges Hypophosphatemia secondary to renal phosphate wasting (in some patients) DD (often speech delay), but normal intelligence		
Craniofrontonasal dysplasia (# 304110)	In females: Coronal synostosis with brachycephaly, frontonasal dysplasia (frontal bossing, widow's peak, low anterior hairline, broad nasal root with bifid nasal tip, hypoplastic nasal tip), telecanthus, strabismus, hypertelorism, cleft lip and/or palate Short and webbed neck, Sprengel deformity scoliosis, unilateral breast hypoplasia, joint laxity Asymmetric lower limb shortness, cutaneous syndactyly, grooved, brittle nails, wiry hair DD, ID, corpus callosum hypoplasia Sensorineural hearing loss Males: hypertelorism, absence of frontonasal dysplasia or other anomalies (more symptoms in case of mosaicism)	*Cellular interference (cell lines with differentially expressed alleles due to random X-inactivation in females)* *EFNB1*	X-linked dominant (both de novo and inherited forms with high variable expressivity in females)
Antley-Bixler syndrome without genital anomalies or disordered steroidogenesis	Multisuture synostosis, brachycephaly, large anterior fontanell, frontal bossing, proptosis, midface hypoplasia, dysplastic ears, depressed nasal bridge	*Gain-of-function* *FGFR2*– p.Ser351Cys	AD (de novo)

(# 207410)	Choanal astresia/stenosis with upper airway obstruction Radiohumeral synostosis, ulnar and femoral bowing, joint contractions, arachnodactyly, camptodactyly, narrow chest and pelvis, rocker-bottom feet DD, ID (variable) Visceral anomalies		
Antley-Bixler syndrome with genital anomalies and disordered steroidogenesis (# 201750)	Similar to the FGFR2-related Antley-Bixler, even cloverleaf skull, tarsal, elbow and/or carpal synostosis Genital anomalies: - females: hypoplastic or fused labia, clitoromegaly, single urogenital orifice, vesico-vaginal fistula, polycystic ovary - males: micropenis, hypospadias, hypoplastic or bifid scrotum, cryptorchidism Disordered steroidogenesis (e.g., increased 17-hydroxyprogesterone	*Loss-of-function (partial)* *POR* – bi-allelic variants	AR
Carpenter syndrome type 1 (#201000)	Multisuture synostosis (sagittal, lambdoid, coronal), brachycephaly, acrocephaly, trigonocephaly, dystopia canthorum, epicanthal folds, flat nasal bridge, missing teeth, delayed shedding of primary dentition Obesity, short thick neck Polysyndactyly of the hands and feet, brachydactyly Scoliosis, absent coccyx, spina bifida occulta, coxa valga, genu valgum, lateral displacement of patellae Heart defects, hydronephrosis, accessory spleens, umbilical hernia, omphalocele	*Loss-of-function* *RAB23* – bi-allelic variants	AR

	DD, ID (variable)		
Carpenter syndrome type 2 (# 614976)	Similar to Carpenter type 1, metopic synostosis (more often) Pectus excavatum, carinatum, hypoplastic and accessory nipples Visceral anomalies with abnormal left-right patterning, hypogonadism in males	*Loss-of-function (partial)* *MEGF8* – bi-allelic variants	AR
Greig cephalopolysyndactyly syndrome (#175700)	Macrocephaly, scaphocephaly with high forehead, frontal bossing, metopic synostosis with trigonocephaly, but also late closing sutures, , hypertelorism, broad nasal root, agenesis of corpus callosum, hydrocephaly Pre- and postaxial polydactyly of hands and feet, halluces, syndactyly 1-3 Mild ID (rare)	*Loss-of-function* *GLI3*	AD (both de novo occurrence and inherited with variable expressivity)
Baller-Gerold syndrome (#218600)	Multisuture (often coronal), turribrachycephaly, micrognathia Absent or hypoplastic thumbs and radii, fused or absent carpal bones, absent metacarpals and/or phalanges, vertebral anomalies Heart defects, renal anomalies, rectovaginal fistula, imperforate or anteriorly placed anus Skin lesions which can evolve into poikiloderma Possibly increased cancer risk (lymphoma) Growth retardation DD, ID	Loss-of-function *RECQL4* – bi-allelic variants affecting the helicase domain which leads to deficient DNA-repair with genomic instability	AR
Shprintzen-Goldberg syndrome (#182212)	Dolichocephaly, often sagittal synostosis, multisuture synostosis, but also large anterior fontanel, high, prominent forehead, maxillary	*Loss-of-function (?)* *SKI* – nuclear protooncogene,	AD (most often de

	hypoplasia, microretrognathia, hypertelorism, telecanthus, proptosis, strabismus, myopia, high palate with prominent palatal ridges, low-set and soft ears Dolichostenomelia with marfanoid habitus, C1-C2 subluxation or fusion, scoliosis, pectus deformity, metatarsus adductus, pes planus, talipes equinovarus, arachnodactyly, camptodactyly, minimal subcutaneous fat, joint laxity and/or contractures, umbilical and inguinal hernia Mitral valves prolapse, aortic root dilatation (rare), arterial tortuosity (rare compared to Loeys-Dietz sd.) DD, ID, hypotonia	repressor of TGBβ-pathway; missense variants with hot-spot in exon 1 (R-SMAD and DHD domains) => increased TGFβ-signaling	novo occurence)
Loeys-Dietz syndrome (LDS) type 1 (#609192) and type 2 (#610168)	Dolichocephaly, often sagittal synostosis, but also coronal, metopic or multisuture synostosis, microretrognathia, malar hypoplasia, hypertelorism, proptosis, strabismus, blue sclerae, bifid uvula, cleft palate Dolichostenomelia with marfanoid habitus, pectus deformity, arachnodactyly, joint laxity, scoliosis, velvety and translucent skin Arterial tortuosity, aortic and cerebral (rare) aneurysm DD, ID (rare)	*Gain-of-function (?)* *TGFBR1* (LDS type 1), *TGFBR2* (LDS type 2) – variants cluster in the intracellular serine-threonine kinase domain of the receptor => increased TGFβ-signaling	AD (most often de novo occurence)
Bohring-Opitz syndrome (C-like syndrome) (#605039)	Trigonocephaly, microcephaly, low frontal hairline, bitemporal narrowing, facial hemangioma (naevus flammeus), microretrognathia, hypertelorism, hypoplastic orbital ridges with prominent eyes with high myopia, strabismus, retinal and optic nerve abnormalities, narrow palate with prominent palatine ridges, cleft lip and palate Typical posture with elbow and wrist flexion, ulnar deviation of the	*Loss-of-function* *ASXL1 (ASXL-transcriptional regulator)*	AD (de novo)

	metacarpophalangeal joints, joint contractures and dislocations, hipertrichosis Heart defects, malrotation, severe gastroesophageal reflux, feeding problems Pre- and postnatal growth retardation Severe DD, ID, seizures, axial hypotonia with hypertonia of the extremities, agenesis/hypoplastic corpus callosum, nodular heterotopia, Dandy-Walker malformation		
C(Opitz trigonocephaly) syndrome (#211750)	Trigonocephaly, microcephaly, epicanthus, up-slanted palpebral fissures, strabismus, short nose with anteverted nares, broad nasal bridge, high arched palate, thick anterior alveolar ridges, oral frenula, macrostomia Pectus deformities, anomalous ribs, scoliosis, radial head and hip dislocation, postaxial polydactyly of hands and feet, metacarpal hypoplasia with ulnar deviation of fingers, skin laxity Visceral anomalies (heart defects, hepatomegaly, renal cysts) Failure to thrive DD, ID, hypotonia, seizures	*Loss-of-function* *CD96 (T-cell activation antigen)*– missense and chromosomal abnormalities such as translocation with breakpoints at 3q13.13	AD (de novo)

AD – autosomal dominant; AR – autosomal recessive; DD – developmental delay; IgII-III-immunoglobulin-like ligand binding domains; ID - Intellectual disability; OMIM - Online Mendelian Inheritance in Man (Catalog of Human Genes and Genetic Disorders); sd. – syndrome