Genome joint analysis in a child at risk for cerebral adrenoleukodystrophy

Bagarić, Matea

UNIVERSITY OF ZAGREB
SCHOOL OF MEDICINE

Matea Bagarić

Genome Joint Analysis in a Child at risk for Cerebral Adrenoleukodystrophy

GRADUATE THESIS

Zagreb, 2024.

This graduate thesis was made at the Center for Innovative and Investigative Pediatric Medicine, Outpatient Pediatric Clinic, Department of Pediatrics, University Hospital Centre Zagreb, and School of Medicine, University of Zagreb under the supervision of Professor Mario Ćuk, MD, PhD, and was submitted for evaluation in the academic year 2023./2024.

ABBREVIATIONS

ABC	Adenosine triphosphate-binding cassette
ABCD1	Adenosine triphosphate-binding cassette subfamily D member 1
AC	Allele count
ACMG	American College of Medical Genetics and Genomics
ACTH	Adrenocorticotropic hormone; adrenocorticotropin
AF	Allele frequency
AI	Artificial intelligence
ALD	Adrenoleukodystrophy
ALDP	Adrenoleukodystrophy protein
AMN	Adrenomyeloneuropathy
AMP	Association for Molecular Pathology
BBB	Blood-brain barrier
C20:0	Arachidic acid
C22:0	Docosanoic acid
C24:0	Tetracosanoic acid
C26:0	Hexacosanoic acid
C26:0-LPC	Hexacosanoic acid-lysophosphatidylcholine
CALD	Cerebral adrenoleukodystrophy
cCALD	Childhood cerebral adrenoleukodystrophy
CGAD	Croatian Genome Aggregated Database; *Genoma*
CGPD	Croatian Genome-Phenotype Database; *FeGena*
CNS	Central nervous system
CoA	Coenzyme A
CRISPR	Clustered regularly interspaced short palindromic repeats
DBS	Dried blood spot
eCDSS	Electronic clinical decision support system
FIA-MS/MS	Flow injection analysis with tandem mass spectrometry
FSH	Follicle-stimulating hormone
GATK	Genome Analysis Toolkit

GEM	Genomic Electronic Medical
gnomAD	Genome Aggregation Database
HPO	Human Phenotype Ontology
HSCT	Hematopoietic stem cell transplantation
HSC	Hematopoietic stem cell
IQ	Intelligence quotient
IVF	In vitro fertilization
IVS	Intervening sequence
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LH	Luteinizing hormone
LoF	Loss-of-function
LS	Lesion score
LV	Lentiviral vector
MRI	Magnetic resonance imaging
NBS	Newborn screening
NIPT	Noninvasive prenatal testing
O/E	Observed/expected
PAI	Primary adrenal insufficiency
PCR	Polymerase chain reaction
PGT	Preimplantation genetic testing
pLI	Loss intolerance probability
UTR	Untranslated region
VCTs	Variant calling tools
VLCFA	Very long-chain fatty acids
VUS	Variant of uncertain significance
WGS	Whole genome sequencing
X-ALD	X-linked adrenoleukodystrophy
XCI	X chromosome inactivation; lyonization

TABLE OF CONTENTS

SUMMARY

Genome Joint Analysis in a Child at risk for Cerebral Adrenoleukodystrophy

Matea Bagarić

Whole genome sequencing (WGS) and genome joint analysis represent a paradigm shift in genetic diagnostics, offering an unparalleled ability to uncover the molecular basis of disease. These innovative, cutting-edge technologies are hailed as first-line diagnostic tools and are particularly critical for patients who remain undiagnosed following exhaustion of conventional medical approaches. Genome joint analysis serves as a biointelligent solution to pinpoint elusive genetic variants, enabling earlier and more precise intervention. Our patient was enrolled in the CROseq Genome Program after traditional diagnostic methods failed to uncover the underlying cause of disease. Joint analysis detected a novel likely pathogenic variant in the *ABCD1* gene in the child. Although he remains in the pre-symptomatic stage, he is currently at the age of highest risk for developing cerebral adrenoleukodystrophy (CALD). In the absence of timely intervention, CALD rapidly progresses to total disability, followed by death, shortly after symptom onset. Early diagnosis is therefore of crucial importance, as hematopoietic stem cell transplantation (HSCT) and *ex vivo* gene therapy can positively alter the disease trajectory and significantly improve patient outcomes when initiated during the pre-symptomatic stage. Establishing the diagnosis during this critical window, however, can be challenging. The biochemical marker, elevated very long-chain fatty acids (VLCFA), is a non-specific finding shared with other disorders of peroxisomal biogenesis. Additionally, the clinical course is complicated by the heterogeneity of clinical manifestations and the potential for cerebral involvement to begin at any age. In this context, genome joint analysis emerges as a powerful tool, enabling the rapid and precise identification of pathogenic *ABCD1* variants and thereby guiding the timely implementation of targeted, life-saving therapies. This review, together with the case presentation, aims to provide a comprehensive overview of adrenoleukodystrophy and highlight the clinical relevance and actionable therapeutic potential of genome joint analysis. The unpredictable clinical course and rapid deterioration observed in CALD underscores the significance of genome joint analysis in providing a comprehensive genetic profile that facilitates the implementation of personalized care for a rare disease with a narrow therapeutic window.

Keywords: genome joint analysis, whole genome sequencing, *ABCD1* gene, cerebral adrenoleukodystrophy

SAŽETAK

Združena analiza genoma u djeteta s rizikom obolijevanja od cerebralne adrenoleukodistrofije

Matea Bagarić

Cijelogenomsko sekvenciranje (WGS) i združena analiza genoma predstavljaju promjenu paradigme u genetičkoj dijagnostici, nudeći neusporedivu sposobnost otkrivanja molekularne osnove bolesti. Ove inovativne, najsuvremenije tehnologije prepoznate su kao dijagnostički alati prve linije i posebno su ključne za pacijente koji ostanu nedijagnosticirani nakon iscrpljivanja konvencionalnim medicinskim metodama. Združena analiza genoma služi kao biointeligentno rješenje za otkrivanje teško prepoznatljivih genetičkih varijanti, omogućujući ranu i preciznu intervenciju. Naš pacijent je uključen u CROseq Genome Program nakon što tradicionalne dijagnostičke metode nisu uspjele otkriti temeljni uzrok bolesti. Združenom analizom je identificirana novo otkrivena vjerojatno patogena varijanta gena *ABCD1* kod djeteta. Iako je i dalje u predsimptomatskom stadiju, trenutno je u dobi s najvećim rizikom za razvoj CALD-a. Bez pravovremene intervencije, CALD brzo napreduje do potpune invalidnosti, praćene smrću, nedugo nakon pojave simptoma. Rana dijagnoza je stoga od presudne važnosti, jer transplantacija hematopoetskih matičnih stanica (HSCT) i genska terapija mogu promijeniti tijek bolesti i značajno poboljšati ishode za pacijente ako se započnu tijekom predsimptomatske faze. Međutim, postavljanje dijagnoze tijekom ovog ključnog razdoblja može biti izazovno. Biokemijski marker, povišene masne kiseline vrlo dugog lanca (VLCFA), nespecifičan je nalaz koji se može naći i u drugim poremećajima peroksisomalne biogeneze. Osim toga, klinički tijek zakompliciran je heterogenošću kliničke slike i mogućnošću zahvaćanja mozga u bilo kojoj dobi. U ovom kontekstu, združena analiza genoma predstavlja korisno sredstvo koje omogućuje brzu i preciznu identifikaciju patogenih varijanti *ABCD1* gena i time omogućuje pravovremenu primjenu ciljanih terapija koje spašavaju život. Cilj je ovoga preglednog članka s prikazom slučaja pružiti sveobuhvatan uvid u adrenoleukodistrofiju i istaknuti kliničku važnost i učinkovitost združene analize genoma. Nepredvidivi klinički tijek s brzim pogoršanjem zdravstvenog stanja kod CALD-a ukazuje na značaj združene analize genoma u pružanju sveobuhvatnog genetskog profila koji olakšava provedbu personalizirane skrbi za rijetku bolest s uskim terapijskim rasponom.

Ključne riječi: združena analiza genoma, cijelogenomsko sekvenciranje, *ABCD1* gen, cerebralna adrenoleukodistrofija

1. INTRODUCTION

Whole genome sequencing (WGS) is an innovative method of molecular genetic testing that offers the most comprehensive view of an individual's genetic variation and is particularly valuable in the identification of rare or novel disease-associated variants that would otherwise go unnoticed by conventional molecular genetic testing or targeted sequencing methods (1). Genome joint analysis builds upon the foundation of WGS by incorporating biointelligent algorithms to analyze not only the patient's genome but also the genetic information from their immediate family members (2). This novel, advanced method enhances diagnostic accuracy by comparing genetic variants across related individuals, allowing for a more nuanced understanding of a variant's pathogenicity (2). In complex cases where the clinical significance of a variant is uncertain, genome joint analysis can provide critical insights into whether a mutation is inherited, arose *de novo*, or is associated with a broader population (3). This multi-dimensional approach enables the early detection of disease-associated variants, facilitates informed decision-making, and paves the way for personalized treatment strategies tailored to each patient, making it an essential tool in proactive patient care and precision medicine.

X-linked adrenoleukodystrophy (X-ALD) is the most prevalent inborn error of peroxisomal metabolism (4). This monogenic disorder arises in the setting of pathogenic alterations in the ATP-binding cassette transporter subfamily D member 1 (*ABCD1*) gene (5). Subsequent deficiency in the adrenoleukodystrophy protein (ALDP) leads to impaired peroxisomal β‑oxidation of saturated very long-chain fatty acids (VLCFA) and their consequent accumulation in tissues and plasma (6). The predominant sites affected are the central nervous system (CNS), adrenal cortex, and testes, corresponding to the four main phenotypes observed: cerebral adrenoleukodystrophy (CALD), adrenomyeloneuropathy (AMN), primary adrenal insufficiency (PAI) and gonadal insufficiency (7). CALD is the most clinically devastating manifestation of X-ALD and develops in approximately one-third of affected males (8). Characterized by a rapidly progressive neurodegenerative course marked by significant impairments in neurologic, cognitive, and behavioral functioning, death can occur within 2 years following symptom onset (5). While the risk of developing cerebral disease is present throughout life, it is highest during childhood, with a peak incidence at 7 years of age (9). Hematopoietic stem cell transplantation

(HSCT) and *ex vivo* gene therapy have the potential to halt disease progression, with a 5-year survival rate of 78% in treated patients compared to 55% in those who remain untreated (10). Both are most effective when initiated prior to onset of neurologic symptoms (11).

Joint analysis is an invaluable method for the timely detection and management of CALD, with 97.5% of pathogenic *ABCD1* variants identified through sequence analysis (12). Through the CROseq Genome Program, trio WGS detected a novel likely pathogenic missense *ABCD1* variant in a 6.5-year-old boy who presented with moderate cognitive impairment, delayed speech and language development, clumsiness, mild autism-like behavior, and various ophthalmologic manifestations. Many *ABCD1* alterations are novel private missense variants often identified in a small population or even in just a single family (5). Consequently, their clinical significance is uncertain, and there are no clear management strategies to determine who may benefit from targeted therapies such as HSCT or *ex vivo* gene therapy. In cases like this, access to a robust genome database is invaluable. The CROseq Genome Program has established two such resources, the Croatian Genome Aggregated Database (CGAD) and the Croatian Genome-Phenotype Database (CGPD), which provide critical insights for variant interpretation. By delivering a comprehensive genetic profile of the proband and their parents, joint analysis enables a more precise, data-driven approach to patient care and provides crucial actionable therapeutic insights that guide personalized management strategies. In rare diseases like CALD, this level of granularity is crucial for tailoring interventions, improving outcomes, and helping to combat the severe impacts of the disease.

2. GENOME JOINT ANALYSIS

Genome joint analysis is an advanced bioinformatics method designed to analyze WGS data from multiple individuals, typically involving family-based trios (proband, mother, and father) or population cohorts (2). Trio-based WGS is especially valuable in diagnosis of rare genetic diseases, as it facilitates the detection of *de novo* variants, a major cause of genetic disorder in outbred populations (13). The primary objective of genome joint analysis is to enhance the precision of genetic variant interpretation, particularly in the context of complex or rare genetic disorders, by leveraging comparative analyses of genetic sequences from related individuals (3).

Key tools employed as part of joint analysis include variant calling tools (VCTs), haplotyping, structural variant callers, and population databases (14–17). VCTs play a central role in identifying genetic variants from raw sequencing data (14). Widely used tools include the Genome Analysis Toolkit (GATK), FreeBayes, and Platypus (14,18). GATK is a robust toolset for variant discovery in high-throughput sequencing data, often used for joint analysis of multiple genomes (14). FreeBayes is an open-source Bayesian genetic variant detector, ideal for both population and trio-based analysis (14). Platypus is a tool that uses localized assembly to detect variants in small genomic regions, increasing the sensitivity to *de novo* alterations (18). Haplotype-based analysis helps infer the likely combinations of alleles inherited from each parent (15). For trio-based WGS, haplotyping determines which genetic variants were inherited from which parent and which arose *de novo* (15). Structural variant callers, such as Manta and Delly, detect larger genetic alterations, including deletions, duplications, and translocations, that may play a role in disease pathogenesis and in this way help capture the full spectrum of genetic changes (16). Finally, population databases, like the Genome Aggregation Database (gnomAD), are instrumental in cross-referencing detected variants with known genetic information, helping to filter out common, benign variants and prioritize rare or novel variants (17,19). Additionally, ClinVar, a repository of clinically significant genetic variants, provides further insights by linking detected variants to specific diseases, enhancing the accuracy of genetic interpretation (20).

Biointelligence and artificial intelligence (AI) play pivotal roles in genome joint analysis by applying sophisticated computational techniques that emulate human reasoning in the interpretation of genetic and genomic data (21). In genome joint analysis, biointelligent algorithms prioritize genetic variants based on their pathogenic potential and identify correlations between genotype and phenotype (21). Predictive modeling algorithms, such as PolyPhen-2, SIFT, and REVEL, assess the functional impact of variants on proteins to predict whether a given amino acid change is likely to be deleterious to protein function (22). AI further enhances these capabilities through advanced variant filtering and interpretation software like SpliceAI, paving the way for a new era of genome interpretation methodology (22). Furthermore, machine learning models are trained on large genetic datasets to classify variants of unknown significance (VUS) into categories of pathogenicity (23). By training on data from population databases like gnomAD and ClinVar, AI algorithms can predict the likelihood that a variant is disease-causing (22).

In the clinical setting, AI systems analyze vast amounts of sequencing data and patient-specific information to assist clinicians in diagnosing and predicting disease trajectories (22). These AI tools integrate clinical, genetic, and bioinformatics data to provide a comprehensive view of a patient's genetic landscape, allowing for precision medicine approaches and improving the accuracy and actionability of medical interventions (22). One example is Fabric Genomic Electronic Medical (GEM), an AI-powered electronic clinical decision support system (eCDSS) used for genetic variant interpretation (24). GEM processes genetic variant calls in VCF format along with case metadata, including parental status and patient phenotypes using Human Phenotype Ontology (HPO) terms (24). GEM integrates multiple variant prioritization algorithms and genomic/clinical databases to score and prioritize potential disease-associated genes through a Bayesian framework (24). It refines variant calls based on genotype quality, sex, ancestry, inheritance patterns, and structural variants, ensuring that the interpretation is contextually accurate (24). GEM's ability to contextualize genetic data showcases how biointelligent and AI-enhanced tools are transforming the accuracy of genetic interpretation and advancing personalized patient care.

3. EPIDEMIOLOGY

X-ALD represents the most common leukodystrophy, with an estimated birth prevalence between 1:14,000 to 1:17,000 newborns (5,7). It is reported worldwide and shows no significant variation in frequency across different ethnicities (25,26). Notably, with a penetrance of 100% in males and 65% in females, X-ALD also stands as one of the most frequent monogenic neurodegenerative disorders (27). The progressive adoption of newborn screening (NBS) programs for X-ALD, coupled with significant advancements in genetic testing, has the potential to reveal a higher true prevalence of this disease.

4. ETIOLOGY AND PATHOPHYSIOLOGY

4.1. MODE OF INHERITANCE

By definition, X-ALD follows an X-linked pattern of inheritance (Figure 1); nevertheless, it can also arise from spontaneous pathogenic alterations that occur in the germ line (28). Approximately 95% of cases stem from a pathogenic variant inherited from one parent, while *de novo* alterations account for an estimated 4% of cases (5,29). Gonadal or gonosomal mosaicism is found in less than 1% of patients (5,29). Heterozygous females have a 50% chance of transmitting the pathogenic *ABCD1* variant to each child in every pregnancy (Figure 1A) (30). A hallmark of X-linked inheritance is the absence of male-to-male transmission, therefore, males hemizygous for a pathogenic *ABCD1* variant will transmit it to all daughters, but not to sons (Figure 1B) (31).

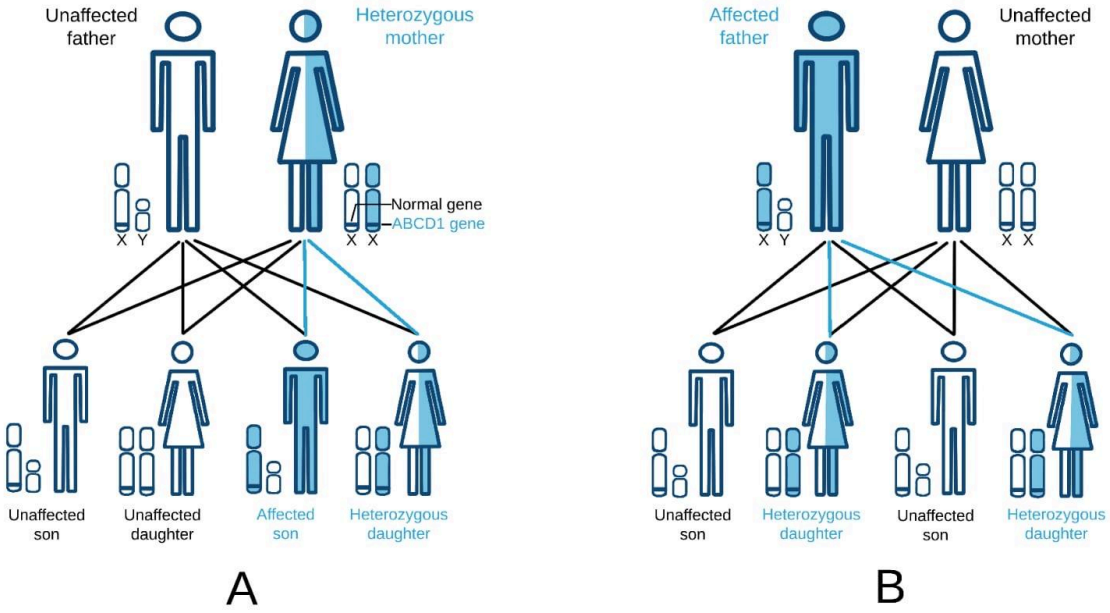

Figure 1. Transmission of the *ABCD1* gene from parents to offspring. **A)** When the mother carries the pathogenic *ABCD1* gene alteration and the father is unaffected, there is a 50% chance with each birth that a son will inherit X-ALD and a 50% chance that a daughter will carry the gene alteration; **B)** When the father has X-ALD and the mother is unaffected, none of the sons will inherit X-ALD, while all daughters will carry the gene alteration, potentially exhibiting symptoms or developing X-ALD.

Created by Lana Zgombić

4.2. GENETIC BASIS

X-ALD is a single-gene disorder caused by pathogenic alterations in the *ABCD1* gene, which is mapped to Xq28, spans 19.9 kb and consists of 10 exons and 9 introns (5,32,33). To date, the 'ABCD1 Variant Database' reports 1227 non-recurrent, unique alterations in the *ABCD1* gene (12). Of these, 647 are missense variants, 131 are nonsense variants, 292 are frameshift variants, 56 are amino acid insertions/deletions, 53 are splice site variants, 25 are one or more exon deletions, 12 are benign variants in the 5' untranslated region (UTR), intervening sequence (IVS), or 3'UTR, and 11 are variants in which no translation was initiated (12).

The *ABCD1* gene encodes ALDP, a 745-amino acid protein that is part of the ATP-binding cassette (ABC) protein transporter family (33). ALDP functions as a transmembrane half-transporter responsible for importing coenzyme A (CoA)-activated VLCFA into peroxisomes for β-oxidation (34). Pathogenic variants in the *ABCD1* gene result in loss of ALDP function, leading to impaired peroxisomal β-oxidation of saturated straight-chain VLCFA (35). In fact, in fibroblasts from patients with X-ALD, the β-oxidation of hexacosanoic acid (C26:0) was diminished to approximately 25% of the levels observed in controls (36). This impairment causes VLCFA to accumulate in plasma and all tissues of the body, with a particular concentration in the white matter of the brain and spinal cord, the adrenal cortex, and the Leydig cells of the testes (37).

4.3. PATHOGENESIS OF CEREBRAL ADRENOLEUKODYSTROPHY

The pathogenesis of CALD is driven by complex, interconnected mechanisms triggered by the accumulation of VLCFA in the brain (38). Elevated levels of VLCFA initiate two key events: (1) destabilization of myelin sheaths leading to their consequent demyelination, and (2) disruption of mitochondrial function and oxidative phosphorylation of neuronal axons (38). These events serve as a catalyst for rapidly progressive inflammatory demyelination, marked by macrophage infiltration and the breakdown of the blood-brain barrier (BBB) (39–41). The earliest and most critical pathogenic event appears to be microglial injury, with a significant reduction in microglial density preceding the degradation of oligodendrocytes and myelin (42). Combined with defective neuroprotection, these processes exacerbate the loss of oligodendrocytes and axons, which is further intensified by an inflammatory response involving altered monocyte function and pro-inflammatory T-cell activity (38). These mechanisms culminate in the pathologic hallmark of CALD, comprised of myelin and axonal loss, oligodendrocyte degeneration, perivascular infiltration of lymphocytes and macrophages, reactive astrocytosis, and migration of reactive microglia to areas of demyelination (43).

5. CLINICAL PRESENTATION

X-ALD is a progressive, clinically heterogeneous disease that affects the nervous system and the adrenal cortex independently (44). Patients are asymptomatic at birth but go on to develop symptoms as the disease advances (45). Phenotypic expression varies across three principal clinical entities: the most severe childhood form, a rapidly progressive, life-threatening leukodystrophy, the most frequent adult form, a slowly progressive myeloneuropathy, and PAI (46). The molecular mechanisms underlying phenotypic variability are poorly understood and modifier genes together with environmental factors are thought to play an important role (27,47). While both male and female patients can develop all of the known phenotypes, affected females classically only develop myeloneuropathy (12). Phenotype and disease course cannot be predicted by the concentration of VLCFA in serum or plasma, the nature of the *ABCD1* variant, or by family history (7). The same pathogenic variant can lead to different phenotypes, and conversely, identical phenotypes may arise from various genetic alterations, including both missense variants that produce abundant immunoreactive protein product and large deletions that result in a complete absence of the gene product (5).

Interestingly, it is not uncommon for diverse phenotypes to be observed within the same family or among siblings (5). Di Rocco et al. (2001) described a case involving 15-year-old monozygotic twin boys diagnosed with X-ALD (48). Despite both twins presenting with elevated VLCFA levels, their clinical manifestations diverged significantly: one twin developed left hemiplegia, seizures, and brain magnetic resonance imaging (MRI) findings consistent with X-ALD, while the other exhibited only adrenal insufficiency, without any neurologic symptoms or abnormalities on neuroimaging (48). Similarly, Sobue et al. (1994) reported phenotypic heterogeneity in adult monozygotic twins with X-ALD; while both developed myeloneuropathy, the older twin showed cognitive impairment with extensive demyelination 10 years earlier than in the younger twin (49). Korenke et al. (1996) documented a case in which CALD developed in only one of a pair of monozygotic twins, despite both sharing an identical X-ALD genotype (50). These findings indicate that factors beyond genetics significantly influence the phenotypic variation observed in X-ALD.

5.1. PHENOTYPIC EXPRESSION IN MALES

Clinically, the primary phenotypes described in boys and men with X-ALD include CALD, AMN, and adrenal insufficiency (Addison disease), which can occur in isolation or in various combinations (51). Gonadal insufficiency, though less common, has also been reported in males with X-ALD (45,52). Some males remain asymptomatic until adulthood, defined as the presence of biochemical and gene abnormality without evident adrenal or neurologic deficit (7). Despite the great variation in clinical presentation, nearly all affected males manifest neurologic symptoms by adulthood (25). Furthermore, the biochemical phenotype of elevated plasma VLCFA has 100% penetrance in males irrespective of age (5). The unpredictability of phenotype development, coupled with the variability in disease onset, progression, and outcomes, underscores the complex nature of X-ALD and the challenges clinicians face in managing the care of these patients.

5.1.1. Cerebral adrenoleukodystrophy

The most severe manifestation of X-ALD is a highly aggressive inflammatory cerebral demyelination marked by focal disruption of the BBB and infiltration of leukocytes into the white matter (53). An estimated one-third of boys with X-ALD go on to develop CALD during their lifetime, with the highest incidence occurring between ages 4 and 10 years, peaking around age 7 years (44,51,54). While the risk factors and pathogenic triggers responsible for the development of these characteristic inflammatory demyelinating lesions are not well understood, the lesions themselves are stereotyped in their anatomic origins, histologic characteristics, and overall progression (54).

Initially, CALD manifests solely as a radiographic finding, with brain MRI lesions appearing long before any clinical symptoms (6,44). Inflammatory demyelination typically begins at the genu or splenium of the corpus callosum and progressively radiates outward in a symmetric, confluent manner in both hemispheres, accompanied by activation of microglial cells, apoptosis, permeability of the BBB, and destruction of axons (27,54). The lesions typically spread across the entire brain and are mirrored by progressive worsening of neurologic ability followed by

eventual death in the absence of timely treatment (54). Early symptoms are non-specific and include learning difficulties, behavioral changes, and attention deficits, which typically become noticeable first in a classroom setting (6,26). In up to 20% of affected boys, seizures are the initial symptom (44). As the disease advances, motor abnormalities, vision impairment, hearing loss, and cognitive decline become apparent (46). This stage is followed by more severe deterioration in neurologic condition, with increased cognitive and behavioral problems, cortical blindness, central deafness, and the onset of quadriparesis (6,44). In rare instances, visual function may be relatively preserved despite significant CNS involvement (44). In its most advanced stage, patients suffer from profound disability and loss of motor function (26). Although the rate of deterioration varies, rapid progression is common (44). Without well-timed intervention and appropriate treatment, CALD invariably leads to rapid neurological decline and death within 2 to 4 years, or leaves patients in a vegetative state for an extended period (46).

CALD may be categorized by age of onset into childhood, adolescent, and adult forms (7). By definition, childhood CALD (cCALD) manifests between 3 and 10 years of age, adolescent CALD between 11 and 21 years, and adult CALD from 22 years onward (7). cCALD is the most common, accounting for 35% to 40% of CALD cases, and is marked by rapid progression and severe neurologic impairment (46). Adolescent CALD, the least common, makes up 4% to 7% of cases and follows a somewhat slower course compared to cCALD, though it can still result in significant neurologic decline (7). Adult CALD represents 20% of cases and has a variable rate of progression. In adults, the onset of AMN symptoms usually precedes cerebral involvement, with long-term follow-up studies indicating that 27% to 63% of patients with AMN develop cerebral symptoms, and 37% to 41% show cerebral demyelination on brain MRI (44). In adult males with CALD, 75% with MRI-detected lesions show involvement of the corticospinal tract, with 50% showing lesions progression (44). Although lesion progression in adults generally occurs more slowly than in children, it remains a progressive process and can be severely debilitating in some cases (44). Adult CALD may present with dementia or other neurologic symptoms, including cognitive decline, impaired coordination, behavioral changes, gait abnormalities, and sensory abnormalities, such as loss of position and vibration sense (26). Despite the distinction between childhood, adolescent, and adult CALD, many authors now view them as variants of cCALD (adolescent-onset X-ALD) and of AMN (adult-onset X-ALD) (26).

In rare cases, a spontaneous halt in the progression of cerebral disease, termed arrested CALD, has been documented (7,27). Mallack et al. (2020) define arrested CALD as the absence of contrast enhancement, no increase in lesion score (LS), and no progression of cerebral symptoms on two or more consecutive MRIs spaced at least six months apart (55). Previously, Korenke et al. (1996) characterized arrested CALD by the lack of neurological deterioration in the absence of lesion progression or contrast enhancement on the latest MRI (56). Of the one-third of boys with X-ALD who develop CALD during childhood, 10% to 15% experience a spontaneous halt in disease progression, known as arrested CALD, without signs of brain inflammation (9). These patients remain stable over several years (55). Arrested CALD lesions can emerge in childhood, with many patients remaining asymptomatic during the early stages (55). Brain lesions often remain clinically silent until the patient presents with adrenal insufficiency or myelopathic symptoms, leading to a diagnosis of X-ALD (55). Overall, the disease progresses slowly over years in arrested CALD, in stark contrast to the rapid neurological deterioration seen in progressive CALD (55). Mallack et al. (2020) studied 22 boys with arrested CALD, revealing that new lesions destined to arrest can develop at the same age as those that will undergo progressive demyelination (55). Additionally, they found that younger children with cerebral disease are more prone to inflammatory demyelination and rapid progression compared to older patients with arrested CALD, who tend to remain stable (55).

5.1.2. Adrenomyeloneuropathy

Myelopathy represents the most common clinical manifestation and primary cause of disability in males with X-ALD (26,57). Nearly all males develop myelopathy, with onset typically between 20 and 40 years of age (44,57). Pathologically, it is characterized by a slowly progressive dying-back axonopathy with atrophy of the spinal cord and peripheral neuropathy (7,27). Clinically, this manifests as progressive stiffness and weakness of the legs due to spastic paraparesis, bladder and bowel dysfunction, impaired sensory perception, particularly of vibration, and sexual dysfunction (5,26,27,51). Peripheral neuropathy symptoms, such as numbness and tingling in the hands and feet, muscle weakness, and loss of coordination and balance, can sometimes be the initial sign of AMN and often go undetected until significant

paraparesis appears (44). On rare occasions, erectile dysfunction may precede motor abnormalities (44). Mobility impairment can become severe enough to require assistive devices or wheelchairs (7). All symptoms develop insidiously over decades (5).

Approximately 80% of males with myelopathy have concomitant adrenal insufficiency and about 60% develop secondary cerebral involvement (57). Adrenal insufficiency is frequently present at the time of AMN diagnosis, sometimes even preceding AMN symptoms by decades (44). While cognitive impairment is generally absent initially, secondary cerebral involvement can occur as the disease progresses and typically arises after the onset of AMN symptoms (26,44). Long-term follow-up studies reveal that 27% to 63% of AMN patients develop cerebral disease, manifesting as cognitive decline, behavioral abnormalities, visual loss, impaired auditory discrimination, or seizures, and 37% to 41% exhibit cerebral demyelination on brain MRI (44). In 10% to 20% of adult males, cerebral involvement leads to rapid neurological decline, serious cognitive and behavioral disturbances, and may result in complete disability and early death (44).

Brain MRI findings in patients with AMN are usually normal, however, spinal cord atrophy can be observed using conventional T2-weighted MRI sequences (44). Total spinal cord area is decreased by 26% to 40% at all levels tested (44). Interestingly, this reduction in spinal cord thickness does not directly correlate with the degree of patient symptoms (44). Instead, sensorimotor abnormalities in the dorsal columns that extend rostrally into the brainstem and internal capsule have been shown to correlate more closely with overall disease severity (44). Patients with AMN do not exhibit the large demyelinating lesions in the CNS typical of CALD, and show minimal to no reactive astrocytosis or lymphocytosis (58). Instead, there is marked symmetric atrophy of the spinal cord, predominantly in the lateral corticospinal, gracile, and spinocerebellar tracts, with distal axonopathy, loss of myelin, and presence of macrophages and gliosis (58). Notably, there is no evidence of active inflammation (58).

5.1.3. Primary adrenal insufficiency

PAI (Addison disease) is a chronic condition of inadequate production of adrenocortical hormones (mineralocorticoids, glucocorticoids, and adrenal androgens) due to a disorder of the

adrenal glands (26). Penetrance of the PAI phenotype in X-ALD has been reported by various research studies to range from 50% to 100%, with lifetime prevalence estimated at over 80% (26,44). In patients with X-ALD, the risk of developing PAI varies by age (26). An international retrospective review examining the medical records of affected boys and men showed that the cumulative probability of developing PAI peaks between ages 3 and 10 years (46.8%), remains significantly elevated until age 40 years (an additional 28.6%), and then sharply declines thereafter (an additional 5.6%) (26,44). Additionally, ALD has been found to be responsible for up to 35% of idiopathic PAI cases for which an autoimmune work-up was inconclusive (44).

PAI has been reported as the initial manifestation of X-ALD in up to 38% of cases (44,59). In contrast to classical PAI, which involves a deficiency of all three adrenal cortex hormones, X-ALD-related PAI primarily impairs glucocorticoid and androgen production due to the preferential accumulation of VLCFA in the zona fasciculata and zona reticularis (44,59). The zona glomerulosa is relatively spared, allowing mineralocorticoid function to remain intact (44,59). In fact, nearly half of patients with X-ALD-related PAI do not develop mineralocorticoid deficiency (44).

Early signs include subclinical abnormalities of glucocorticoid secretion, detectable as early as the fifth week of life (44,59). Clinically, elevated adrenocorticotropin (ACTH) levels manifest as hyperpigmentation of areas not usually exposed to sunlight, such as the palmar creases and mucous membrane of the oral cavity (59). Hypocortisolism manifests with fatigue, lethargy, weight loss, anorexia, gastrointestinal complaints (nausea, vomiting, diarrhea), muscle aches, weakness, sugar cravings, and orthostatic hypotension (59). Severe deficiency of glucocorticoids may precipitate a life-threatening adrenal crisis (Addisonian crisis), necessitating prompt treatment and monitoring (59). Individuals that develop mineralocorticoid deficiency present with hypotension, dizziness, salt craving, electrolyte imbalances (hyponatremia, hyperkalemia, metabolic acidosis), decreased plasma aldosterone, and increased plasma renin activity (59).

Cytotoxicity of VLCFA in the zona fasciculata and zona reticularis is thought to culminate in apoptosis and subsequent atrophy of the adrenal cortex (26,44). The natural progression of adrenocortical insufficiency in X-ALD remains unclear due to the absence of comprehensive

prospective studies (57). However, the decline in adrenal function is gradual, initially beginning with progressive elevations in ACTH and eventually resulting in an abnormal cortisol response to an ACTH stimulation test and onset of endocrine symptoms (44,57). Abnormal adrenal function is observed in 90% of neurologically symptomatic boys with X-ALD and in 70% of men with AMN (26).

5.1.4. Gonadal insufficiency

Gonadal insufficiency, marked by a gradual deterioration in the function of Leydig cells, decreased testosterone levels, and increased luteinizing hormone (LH) and follicle-stimulating hormone (FSH), has been described in boys and men with X-ALD (26,45). Clinically, patients may present with delayed puberty, reduced lean muscle mass, low bone mineral density, lack of libido, erectile dysfunction, mood changes, and fatigue (59). The significance of these findings is not yet clear; for example, impotence may indicate primary gonadal insufficiency or may be a reflection of spinal cord disease (45). Fertility is generally preserved in the early, pre-symptomatic stage, but deteriorates progressively with disease advancement (26,59).

5.2. PHENOTYPIC EXPRESSION IN FEMALES

Heterozygous females with pathogenic *ABCD1* variants exhibit an age-related risk of developing X-ALD; girls remain symptom-free during childhood, while 18% of women under 40 years and 88% of women over 60 years develop symptoms (5,6,60). Clinically, the primary phenotype described in women with X-ALD is AMN (7,26,44). Extremely rarely, cases of CALD and PAI have been documented in females (7,26,44).

X chromosome inactivation (XCI) is thought to explain symptomatic X-ALD in female carriers (6,26,61). Maier et al. (2002) reported that symptom manifestation in 22 heterozygous females was linked to skewed XCI, with a significant correlation between the degree of skewing and the severity of neurological abnormalities (61). Conversely, Watkiss et al. (1993) and Salsano et al. (2012) found no association between neurological manifestations and XCI patterns (62,63).

5.2.1. Cerebral adrenoleukodystrophy

CALD is believed to only develop in homozygous females or in the presence of complete XCI (44). In the few documented cases, the development of CALD in women has been linked to skewed X inactivation favoring the X chromosome carrying the pathogenic variant or to a deletion of the normal X chromosome at Xq27-ter (7,61,64).

5.2.2. Adrenomyeloneuropathy

The reported incidence of AMN in heterozygous females varies between 65% and 88% (6,57,60). Symptom prevalence increases with age, with estimates suggesting that about 50% of women over 40 and approximately 65% of women by age 65 will develop symptoms (7). Onset of myeloneuropathy occurs later in females compared to males and progression is more gradual (7). Symptoms typically begin in the fourth decade of life and include mild spasticity, bladder and bowel incontinence, and gait difficulties (44). About 90% of women develop neuropathic pain during their lifetime, a symptom not commonly observed in males (44).

5.2.3. Primary adrenal insufficiency

Adrenal insufficiency is a rare finding in heterozygous females, with reported prevalence rates ranging from 1% to 5% (7,26,65,66). In contrast to males, PAI does not precede the onset of the AMN phenotype in females (7).

6. DIAGNOSTIC TECHNIQUES

To establish a diagnosis of X-ALD, two criteria must be satisfied: the detection of abnormally elevated VLCFA in serum or plasma and the identification of a pathogenic or likely pathogenic variant in the *ABCD1* gene (5). Since newborns with ALD appear neurologically normal at birth and the therapeutic window for CALD is narrow, early diagnosis is crucial for initiating life-saving treatments and improving outcomes for affected individuals.

6.1. ADVANCED, INNOVATIVE, AND BIOINTELLIGENT METHODS

6.1.1. The CROseq Genome Program

The CROseq Genome Program is a pioneering collaborative effort between Croatian institutes and Brigham and Women's Hospital (Boston, USA), funded by the Mila Za Sve Foundation. As the first CGAD and first CGPD, it tackles the challenges that genetic diagnosis of Mendelian diseases pose in the pediatric population in Croatia. CROseq uses the latest state-of-the-art genomic analysis tools together with AI to analyze complex and rare diseases by joint genome analysis of the affected child and their family members to uncover disease etiology.

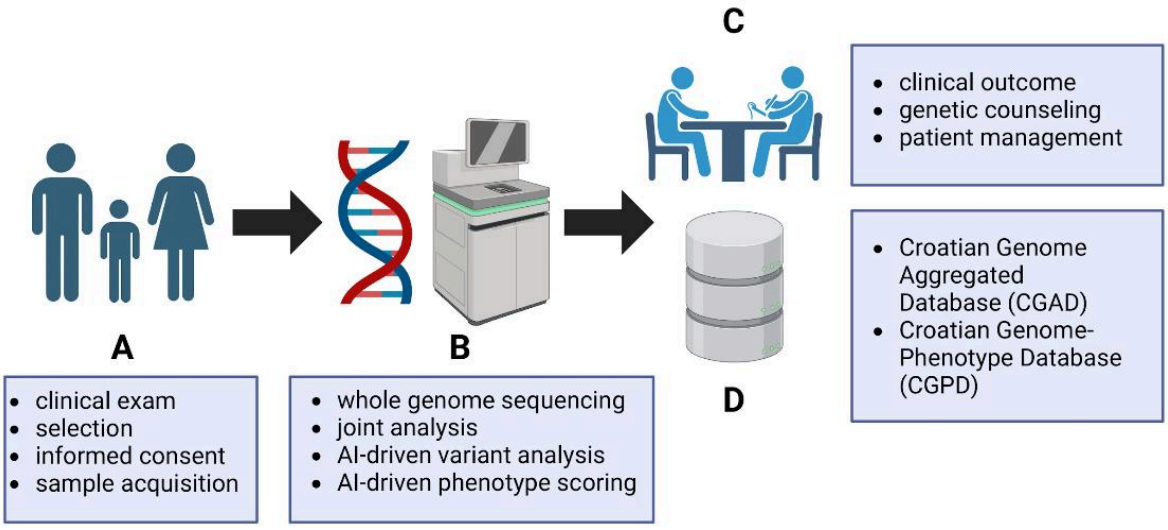

Figure 2. The CROseq Genome Program pipeline. **A)** Clinical examination was performed in Croatian participants, consent was obtained from suitable candidates who were then enrolled in

the program, and phenotypes were established at the Department of Pediatrics, University Hospital Centre Zagreb; **B)** Samples were sent to the USA for WGS and analysis. The whole nuclear genome and mitochondrial DNA were sequenced and joint analysis of the affected proband and unaffected parents was performed. AI-driven analytical tools were used for phenotype scoring, variant prioritization, and variant classification; **C)** Clinical decision-making and patient management strategies were performed by the CROseq expert team, consisting of attending physicians and medical geneticists; **D)** CGAD and CGPD were developed by the CROseq computational group, comprised of computational, genomic, and clinical experts.
Created with Biorender.com

A total of 55 million genomic variants have been analyzed from 754 participants enrolled in the CROseq Genome Program. Clinical and pathogenic assessment of genetic variants is conducted using the CGAD and CGPD databases to determine the population-specific allele count (AC) and allele frequency (AF) of the variant in question.

6.1.2. Whole genome sequencing

WGS is the gold standard for the diagnosis of X-ALD (1). In comparison to other methods of molecular genetic testing, WGS detects a broader range of genomic variations, offering a comprehensive genetic testing solution that can simultaneously explore both coding and non-coding regions of the genome, and thereby removes the necessity for performance of sequential genetic testing (67,68).

The analytic approach for WGS can be either genotype-driven, focusing on identifying pathogenic variants, or phenotype-driven, targeting variants consistent with disease inheritance patterns (69). These approaches are often combined, and for diseases with well-defined symptoms, in silico gene panels can be used early in the analysis to narrow the focus (1). The strategy utilized depends on the clinical presentation and whether the case is isolated or has a familial predisposition (1). In children with unaffected parents, trio sequencing, in which the child and both parents are analyzed, increases diagnostic accuracy by identifying *de novo* heterozygous or compound heterozygous variants consistent with the clinical diagnosis (70). Trio

analyses are more successful than singleton analyses, typically requiring the evaluation of only 10 to 30 variants (71).

The American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) (ACMG/AMP) classification system is commonly used to prioritize variants based on pathogenicity (72). Variants are categorized into five groups: benign, likely benign, VUS, likely pathogenic, and pathogenic (72). Studies of WGS in patients with rare diseases report an average diagnostic yield of around 25% (73). Notably, over 10% of these diagnoses involved variants located in genomic regions that other methods could not detect, while a small percentage were coding variants missed due to low coverage in exome sequencing (1,74). Wright et al. (2023) demonstrated that in pediatric disorders, WGS diagnoses about 40% of probands, with 76% of those involving *de novo* pathogenic variants (71). Diagnostic success is highest in trio analyses and among patients with pronounced symptoms (71). Children with intellectual disabilities, neurodevelopmental disorders, and complex syndromes benefit from early WGS, which can also uncover unique presentations of known diseases or entirely new syndromes, making it an invaluable tool for advancing disease classification and discovery of new genetic conditions (75–77).

6.2. TRADITIONAL METHODS

6.2.1. Biochemical testing

Biochemical testing for X-ALD involves measuring the concentration of VLCFA in either serum or plasma (5). The assay evaluates three parameters: the levels of hexacosanoic acid (C26:0) and tetracosanoic acid (C24:0), the ratio of C26:0 to docosanoic acid (C22:0), and the ratio of C24:0 to C22:0 (32,44). Characteristically, C26:0 is the most consistently elevated and is therefore of the highest diagnostic utility (32,43). In some centers, the derivative species C26:0-lysophosphatidylcholine (C26:0-LPC) is also measured, with a sensitivity greater than 99% (5,59). False elevations in VLCFA have been reported in cases of liver insufficiency or in patients on ketogenic diets (32). Additionally, false-negative results occur in up to 20% of female

patients, and thus symptomatic females, regardless of family history, should always undergo further genetic testing (32,59).

Importantly, while VLCFA testing is highly sensitive, the biomarkers used are nonspecific and cannot distinguish ALD from other disorders of peroxisomal β-oxidation (44,59). Therefore, genetic testing of the *ABCD1* gene is necessary to make a definitive diagnosis (59).

6.2.2. Imaging

Brain MRI is indicated in all male patients, even in the absence of cognitive or neurologic symptoms (45). Ideally, a baseline MRI should be performed at 2 years of age, followed by screening every 6 months until age 12 years, and annually thereafter (45). Routine screening for CALD in females, regardless of age, is not recommended (45). In adult males, surveillance with MRI should be continued as long as HSCT remains a therapeutic option (45).

6.2.3. Newborn screening

NBS for X-ALD is performed by measuring the concentration of C26:0-LPC in dried blood spot (DBS) samples and serves as a highly sensitive screening method (5,7,59). Most NBS programs adopt a standardized three-tier approach for ALD testing, typically involving two biochemical assessments followed by confirmatory genetic testing (59).

To date, NBS for X-ALD is conducted in the United States, Taiwan, and the Netherlands (5,59,78,79). Pilot projects or regional screening initiatives are underway in Italy, Spain, Slovenia, Ireland, Israel, and Japan (51,59,80). In the United States, X-ALD was added to the Recommended Uniform Screening Panel in 2016, and currently, 30 states and Washington, DC have incorporated this screening into their healthcare systems (44,59). The Netherlands began implementing sex-specific screening of newborn boys in 2021 (44,59).

Significant efforts are underway to integrate newborn genetic screening with traditional NBS, which would enable early detection, monitoring, and intervention of genetic disorders before the

onset of symptoms (81,82). Initiatives like BeginNGS are working to scale newborn genetic screening globally by harnessing the power of advanced genome sequencing technologies to identify conditions that might otherwise go undetected by traditional and conventional screening methods (81). These efforts are poised to transform early diagnosis and intervention for rare genetic diseases.

6.2.4. Prenatal testing

Prenatal testing may be performed in women with a positive family history for X-ALD or in those who have given birth to an affected child (59). Traditionally, polymerase chain reaction (PCR) sequencing following amniocentesis or chorionic villus sampling has been done (59). Today, preimplantation genetic testing (PGT), a technique used in conjunction with in vitro fertilization (IVF), can be conducted to screen embryos for ALD prior to implantation (44,59). Moreover, the future of prenatal ALD diagnosis is expected to expand with the increased utilization of noninvasive prenatal testing (NIPT) using cell-free fetal DNA (59). While NIPT has traditionally been employed to detect fetal aneuploidies, recent advancements have broadened its application to include single-gene disorders, positioning ALD as a promising candidate for its implementation (44).

7. TREATMENT

7.1. HEMATOPOIETIC STEM CELL TRANSPLANTATION

HSCT is the preferred treatment for early ALD (59). Eligible candidates have a Loes score ≤ 9 and a neurological score of 0 or 1 (59). Research indicates that HSCT achieves optimal outcomes when performed on asymptomatic individuals exhibiting minimal but characteristic MRI findings of early-stage cCALD (5). These findings commonly manifest as small T2 hyperintensities primarily located in the splenium or genu of the corpus callosum, occasionally extending to the corticospinal tracts or cerebellar white matter (5). Conversely, HSCT is not advised for males lacking cerebral involvement on MRI, as approximately 50% are likely to remain disease-free (59). The procedure typically involves allogeneic stem cell transplantation

using donors such as matched-sibling donors (with the lowest risk), unrelated matched donors, or umbilical cord blood (5). In X-ALD, HSCT has demonstrated overall survival rates of 82% at two years and 74% at five years post-transplant, compared to a 55% survival rate at five years for untreated cCALD (5). Importantly, HSCT does not reverse neurological changes, prevent adrenal involvement, or address myeloneuropathy, and has not shown efficacy in advanced cCALD (59).

7.2. GENE THERAPY

For patients lacking a suitable donor, gene therapy utilizing genetically modified autologous hematopoietic stem cells (HSC) offers an alternative (59). Viral vectors, adept at circumventing preexisting immunity, facilitate precise targeting and efficient transduction of therapeutically relevant cells, ensuring sustained genome maintenance and appropriate expression of transgenes (83). Infusion of modified HSC that express the wild-type lysosomal enzyme holds promise for ameliorating the severe CNS degeneration in CALD (83). Early trials with ALD patients who received HSC transduced with lentiviral vectors (LV) pseudotyped with vesicular stomatitis virus glycoprotein and expressing the *ABCD1* gene demonstrated halted brain demyelination at 14 and 20 months post-therapy, with no new lesions observed 36 months later (83). The successful outcomes from phase I/II and phase III trials led to the approval of the LV-based therapy Skysona for cerebral ALD in 2022 (83).

Recently approved in the United States, Skysona, also known as elivaldogene autotemcel, employs *ex vivo* gene therapy (5). This treatment involves transfecting patient-derived hematopoietic precursor cells with a functional copy of the *ABCD1* gene using a lentiviral approach (5). Eligibility criteria include early cCALD evident on contrast-enhanced MRI, absence of neurological signs, and a performance intelligence quotient (IQ) exceeding 80 from neuropsychological testing, alongside the unavailability of a matched-sibling donor (5). This approach offers potentially reduced risks of graft-versus-host disease compared to HSCT (5). However, it carries inherent risks, notably the possibility of hematologic malignancies such as myelodysplastic syndrome due to the integration of the lentiviral vector, Lenti-D, into proto-oncogenes (5). Consequently, ongoing monitoring, with biannual complete blood counts during the first year and annual assessments thereafter, is imperative (5).

7.3. GENE EDITING

Gene editing techniques have shown success both *in vitro* with ALD patient-derived fibroblasts and *in vivo* in mice (59). In murine models, intravenous delivery of two adeno-associated virus 9 vectors—one encoding clustered regularly interspaced short palindromic repeats (CRISPR) and another carrying a homology-independent targeted integration donor—resulted in the targeted integration of the human *ABCD1* gene. This intervention notably increased *ABCD1* mRNA levels and decreased plasma VLCFA levels (59). Thus, gene editing holds significant promise as a prospective treatment option for the future (59).

8. CASE PRESENTATION

The patient was born extremely preterm at 23 weeks' gestation via vaginal delivery from the mother's first pregnancy. At birth, he weighed 640 grams, measured 29 cm in length, and had an Apgar score of 2/4. At 5.5 years of age, he presented with developmental delay, significant delay in speech and language development, moderate cognitive impairment, minor neuromotor dysfunction, clumsiness, mild autism-like behavior, and a range of ophthalmologic manifestations, including myopia, esotropia, latent nystagmus, and amblyopia. Family history is negative for X-ALD. The parents are non-consanguineous and both the mother and father are healthy. Despite exhaustive efforts involving all possible traditional, conventional, and standard diagnostic methods, the underlying etiology remained elusive and undetected in this patient.

8.1. GENOME JOINT ANALYSIS

In this setting, the patient and his family were enrolled in the CROseq Genome Program and trio-based WGS was performed in the proband and his parents.

HPO terms related to the patient's clinical presentation were incorporated into the analysis pipeline. These included 'extremely preterm birth', 'cognitive impairment', 'delayed speech and language development', 'expressive language delay', and 'clumsiness'. A thorough examination of variants across all genomic regions was performed, focusing on high and medium confidence variants while excluding those with low confidence or that failed technical quality checks. Variants were prioritized based on their relevance to the proband's clinical findings, and phasing analysis was employed towards possible disease-causing variants.

The ACMG/AMP guidelines were used for variant classification. AF data from gnomAD (v2.1.1) was assessed using a gene-specific threshold. For protein-coding variants, in silico predictions were done with tools such as SIFT, Polyphen, Mutation Assessor, MutationTaster, FITCONS, FATHMM, GENOCANYON, dbscSNV RF, and dbscSNV ADA, with threshold scores set at 0.7 for deleterious effects and 0.15 for benign effects. Splice site variants were evaluated using SpliceAI, with thresholds of 0.7 for high, and 0.2 for low, confidence.

Loss-of-function (LoF) intolerance was determined with a loss intolerance probability (pLI) equal to 1 and an observed/expected (O/E) score less than 0.35. Additionally, gene-phenotype relationships were assessed using ClinVar submissions and online resources such as GeneReviews, OMIM, PubMed, and Orphanet.

Phenotype-driven trio WGS of the patient and his unaffected parents identified a novel NM_000033.4(*ABCD1*):c.1145C>T;p.Thr382Ile missense variant in the child and his mother. According to ACMG criteria, this variant is classified as likely pathogenic (PM2, PM5, PP2, PP3). At the time of writing, this variant has neither been reported to ClinVar or gnomAD, nor has it been described in the scientific literature. However, unpublished data from the Moser Center for Leukodystrophies at the Kennedy Krieger Institute indicate that a variant at the same codon position, but with a different amino acid change (p.Thr382Arg), has been observed in one X-ALD case, and this variant was classified as likely pathogenic.

8.2. FURTHER INVESTIGATIONS

8.2.1. Biochemical testing

Following identification of a variant in the *ABCD1* gene classified as likely pathogenic, the subsequent step in the evaluation involved assessment of the levels of VLCFA in the patient. The results are summarized in Table 1. Although the C26:0 level was elevated, the acylcarnitine and LPC levels were not suggestive for X-ALD.

Table 1. Summary of biochemical test results.

Abbreviations: FIA-MS/MS, flow injection analysis with tandem mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry

Parameter	Result	Units	Reference Range	Method
C20:0	0,04	µmol/L	0,01 - 0,11	FIA-MS/MS
C22:0	0,02	µmol/L	0,01 - 0,02	FIA-MS/MS
C24:0	**0,06** H	µmol/L	0,01 - 0,04	FIA-MS/MS

C26:0	**0,07** H	µmol/L	0,01 - 0,03	FIA-MS/MS
C20:0-LPC	**1,03** H	µmol/L	0,09 - 1,00	FIA-MS/MS
C22:0-LPC	**0,49** H	µmol/L	0,08 - 0,46	FIA-MS/MS
C24:0-LPC	0,65	µmol/L	0,18 - 0,70	FIA-MS/MS
C26:0-LPC	0,5	µmol/L	0,10 - 0,50	FIA-MS/MS
C26:0	**0,07** H	µmol/L	0,00 - 0,03	LC-MS/MS
C26:0-LPC	0,08	µmol/L	0,00 - 0,10	LC-MS/MS

8.2.2. Magnetic resonance imaging

At 5 months of age, brain MRI revealed a slightly turricephalic and plagiocephalic skull shape. There was reduced volume of the vermis and the left cerebellar hemisphere, which resulted in a wide communication between the fourth ventricular and pericerebellar cerebrospinal fluid spaces. The supratentorial organization of the gyri and sulci appeared normal. The pattern of myelination was appropriate for age, and the corpus callosum was normally shaped but slightly reduced in volume. A punctate zone of degradation products from earlier hemorrhage was present on the right side in the caudothalamic groove, with minimal hemosiderin deposits in the occipital horn of the left lateral ventricle. There were no signs of acute ischemia, fresh bleeding, expansile processes, or hydrocephalus. The optic chiasm, cavernous sinuses, pontocerebellar angles, and cranio-cervical junction were within normal morphologic limits.

At 6.5 years of age, follow-up brain MRI revealed an oval T2/FLAIR hyperintense lesion, approximately 4 mm in diameter, in the right cerebral peduncle. Two smaller oval lesions of similar characteristics were found bilaterally in the posterior limbs of the internal capsule. Myelination has remained appropriate for age. The corpus callosum was well-developed in all segments but had a slightly reduced volume. The ventricular system was midline, with moderately enlarged lateral ventricles without signs of hypertensive hydrocephalus. No fresh ischemia or hemorrhage was detected, nor any focal expansile processes. The cerebellar

hemispheres were found to be asymmetrical, with the left hemisphere moderately reduced in volume and surrounded by a wider cerebrospinal fluid space. There was broad communication between the fourth ventricle and the cisterna magna. The ocular bulbs were bilaterally atypically shaped with pronounced anteroposterior diameters. Mild plagiocephaly was noted.

Spinal MRI at 6.5 years of age showed normal vertebral body height and preserved posterior intercorporal line. No herniation of intervertebral discs or spinal developmental anomalies were observed. The spinal cord appeared of normal morphology and signal intensity, with no signs of atrophy. The spinal canal width was appropriate, and the conus medullaris was in its normal position. No intradural or extradural focal processes were detected.

9. DISCUSSION

WGS has revolutionized genetic diagnostics by providing a groundbreaking, comprehensive method for uncovering the molecular underpinnings of disease. It is particularly valuable in the diagnosis of monogenic disorders and rare genetic conditions, offering an unparalleled profile of an individual's entire genome and far surpassing the capabilities of traditional and conventional testing approaches. Genome joint analysis is a biointelligent tool that builds upon WGS to further refine the precision of variant interpretation. By enhancing the ability to detect *de novo* variants and assess their pathogenicity, joint analysis equips clinicians with a clearer understanding of disease risks and guides more informed, targeted intervention strategies.

The CROseq Genome Program is a pioneering initiative in Croatia designed to harness the power of WGS and genome joint analysis to address rare diseases in the Croatian pediatric population. The CGAD and CGPD databases established through the CROseq Genome Program are valuable resources for classifying novel variants that have not yet been reported in the literature. While one such detected variant may initially be classified as pathogenic or likely pathogenic based on ACMG criteria, a thorough cross-analysis with population-based databases that evaluate AC and AF can provide additional insights into the pathogenicity of a newly discovered variant. The clinical significance and actionable potential of genome joint analysis paired with population-based databases lie in their capacity to facilitate the timely detection of disease-associated variants and to guide personalized patient care. By creating a comprehensive genetic roadmap for individualized care, joint WGS enables proactive decision-making tailored to each patient's unique risk profile.

As part of the CROseq Genome Program, trio joint analysis identified a novel likely pathogenic missense variant in the *ABCD1* gene in a 6.5-year-old boy and his mother. Detection of this disease-associated variant while the patient is still in the presymptomatic stage has been pivotal in guiding ongoing care and follow-up. Awareness that the patient is at risk for developing CALD, particularly given his age when rapid deterioration is commonly observed, allows for vigilant, guideline-directed monitoring. This involves frequent neurologic assessments and contrast-enhanced brain MRI scans every six months. Should MRI findings indicate the onset of

CALD, discussions regarding the initiation of HSCT or *ex vivo* gene therapy can be promptly addressed. This transformative approach represents a shift from reactive to proactive care in the management of X-ALD.

Going forward, the next step in management of this patient involves analysis of the 754 genomes collected through the CROseq Genome Program utilizing the CGAD and CGPD databases to determine whether the identified variant is present in other individuals within the Croatian population. This analysis provides a unique opportunity to refine our understanding of the variant's pathogenicity. If the variant is found in healthy individuals without signs or symptoms of X-ALD, it may necessitate a reassessment of its classification and a subsequent adjustment of the management plan. This capability to further investigate variant pathogenicity highlights the value of genome databases in personalizing medical care and developing actionable management strategies tailored to the individual patient.

Looking to the future, expanding population-based genetic databases like CGAD and CGPD will significantly enhance the precision of variant interpretation in the Croatian population. The CROseq Genome Program stands as the first large-scale whole genome initiative in Croatia, specifically focused on addressing rare and complex diseases in the pediatric population. This program is not only a crucial resource for genetics and population-based research within Croatia but also holds value for studies beyond its borders. Croatian genomic datasets contribute to variant prioritization strategies and classification, advancing understanding of the genetic basis of rare diseases, and to disease-associated gene discovery.

10. CONCLUSION

Genome joint analysis holds the potential to revolutionize both the diagnosis and management of CALD. By integrating genomic tools such as WGS with population-based databases like CGAD and CGPD, a more detailed understanding of genetic variants and their implications for disease management can be achieved. This integration enhances the physician's ability to personalize care, anticipate the onset of severe phenotypes, and ultimately support better long-term outcomes for children at risk of developing CALD. Initiatives such as the CROseq Genome Program not only advance our scientific understanding of rare diseases but also deliver practical benefits for patient care, especially in pediatric populations.

11. ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my mentor, Professor Mario Ćuk, MD, PhD. His unwavering support and invaluable guidance have been a cornerstone of my academic growth, and I am truly honored to have the privilege and fortune of working with someone as inspiring and visionary. Thank you for believing in me and for setting a standard of excellence that I will carry with me always.

With heartfelt thanks to my dear friend, Lana Zgombić, for her exceptional artistry in crafting the inheritance pattern figure and sprinkling her creative magic into my thesis.

To my mother, Snježana Soldo, whose example of what it means to be a remarkable woman has been a guiding light. Your strength and resilience have emboldened me to embrace my own path with confidence and courage.

To my late grandmother, Šimka Soldo, who was my steadfast companion and best friend. Your enduring love and support continue to be a profound presence in my life every day.

To my beloved dog, Nala, who was the best younger sister a girl could ask for. I love you with all my heart. May your wings carry you to places your feet couldn't.

This thesis is dedicated to my late father, Stipan Bagarić.
In the words of Marko Perković Thompson, *samo je ljubav tajna dvaju svjetova*.

12. REFERENCES

1. Bagger FO, Borgwardt L, Jespersen AS, Hansen AR, Bertelsen B, Kodama M, et al. Whole genome sequencing in clinical practice. BMC Med Genomics. 2024 Jan 29;17(1):39.

2. Bar O, Vahey E, Mintz M, Frye RE, Boles RG. Reanalysis of Trio Whole-Genome Sequencing Data Doubles the Yield in Autism Spectrum Disorder: De Novo Variants Present in Half. Int J Mol Sci. 2024 Jan 18;25(2):1192.

3. Radoi VE, Pop LG, Maioru OV, Dan A, Riza M, Novac MA, et al. CUTTING EDGE TRIO-WGS IN RARE GENETIC SYNDROME DIAGNOSIS. Acta Endocrinol Buchar Rom 2005. 2022;18(4):541–4.

4. Manor J, Chung H, Bhagwat PK, Wangler MF. ABCD1 and X-linked adrenoleukodystrophy: A disease with a markedly variable phenotype showing conserved neurobiology in animal models. J Neurosci Res. 2021 Dec;99(12):3170–81.

5. Raymond GV, Moser AB, Fatemi A. X-Linked Adrenoleukodystrophy. In: Adam MP, Feldman J, Mirzaa GM, Pagon RA, Wallace SE, Bean LJ, et al., editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993 [cited 2024 Sep 8]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK1315/

6. Li Z, Lai G. X-linked adrenoleukodystrophy caused by maternal ABCD1 mutation and paternal X chromosome inactivation. Exp Ther Med. 2022 Sep;24(3):565.

7. Turk BR, Theda C, Fatemi A, Moser AB. X-linked adrenoleukodystrophy: Pathology, pathophysiology, diagnostic testing, newborn screening and therapies. Int J Dev Neurosci Off J Int Soc Dev Neurosci. 2020 Feb;80(1):52–72.

8. Mallack EJ, Van Haren KP, Torrey A, van de Stadt S, Engelen M, Raymond GV, et al. Presymptomatic Lesion in Childhood Cerebral Adrenoleukodystrophy: Timing and Treatment. Neurology. 2022 Aug 1;99(5):e512–20.

9. Mallack EJ, Askin G, van de Stadt S, Caruso PA, Musolino PL, Engelen M, et al. A Longitudinal Analysis of Early Lesion Growth in Presymptomatic Patients with Cerebral Adrenoleukodystrophy. AJNR Am J Neuroradiol. 2021 Oct;42(10):1904–11.

10. Raymond GV, Aubourg P, Paker A, Escolar M, Fischer A, Blanche S, et al. Survival and Functional Outcomes in Boys with Cerebral Adrenoleukodystrophy with and without Hematopoietic Stem Cell Transplantation. Biol Blood Marrow Transplant J Am Soc Blood Marrow Transplant. 2019 Mar;25(3):538–48.

11. Mallack EJ, Turk B, Yan H, Eichler FS. The Landscape of Hematopoietic Stem Cell Transplant and Gene Therapy for X-Linked Adrenoleukodystrophy. Curr Treat Options Neurol. 2019 Nov 25;21(12):61.

12. Mallack EJ, Gao K, Engelen M, Kemp S. Structure and Function of the ABCD1 Variant Database: 20 Years, 940 Pathogenic Variants, and 3400 Cases of Adrenoleukodystrophy. Cells. 2022 Jan 14;11(2):283.

13. Lappalainen T, Scott AJ, Brandt M, Hall IM. Genomic Analysis in the Age of Human Genome Sequencing. Cell. 2019 Mar 21;177(1):70–84.

14. Zverinova S, Guryev V. Variant calling: Considerations, practices, and developments. Hum Mutat. 2022 Aug;43(8):976–85.

15. Glusman G, Cox HC, Roach JC. Whole-genome haplotyping approaches and genomic medicine. Genome Med. 2014;6(9):73.

16. Mahmoud M, Gobet N, Cruz-Dávalos DI, Mounier N, Dessimoz C, Sedlazeck FJ. Structural variant calling: the long and the short of it. Genome Biol. 2019 Nov 20;20(1):246.

17. Gudmundsson S, Singer-Berk M, Watts NA, Phu W, Goodrich JK, Solomonson M, et al. Variant interpretation using population databases: Lessons from gnomAD. Hum Mutat. 2022 Aug;43(8):1012–30.

18. Rimmer A, Phan H, Mathieson I, Iqbal Z, Twigg SRF, WGS500 Consortium, et al. Integrating mapping-, assembly- and haplotype-based approaches for calling variants in clinical sequencing applications. Nat Genet. 2014 Aug;46(8):912–8.

19. Król ZJ, Dobosz P, Ślubowska A, Mroczek M. WGS Data Collections: How Do Genomic Databases Transform Medicine? Int J Mol Sci. 2023 Feb 3;24(3):3031.

20. Landrum MJ, Lee JM, Benson M, Brown GR, Chao C, Chitipiralla S, et al. ClinVar: improving access to variant interpretations and supporting evidence. Nucleic Acids Res. 2018 Jan 4;46(D1):D1062–7.

21. Vilhekar RS, Rawekar A. Artificial Intelligence in Genetics. Cureus. 2024 Jan;16(1):e52035.

22. Lin Q, Tam PKH, Tang CSM. Artificial intelligence-based approaches for the detection and prioritization of genomic mutations in congenital surgical diseases. Front Pediatr. 2023;11:1203289.

23. Quazi S. Artificial intelligence and machine learning in precision and genomic medicine. Med Oncol Northwood Lond Engl. 2022 Jun 15;39(8):120.

24. De La Vega FM, Chowdhury S, Moore B, Frise E, McCarthy J, Hernandez EJ, et al. Artificial intelligence enables comprehensive genome interpretation and nomination of candidate diagnoses for rare genetic diseases. Genome Med. 2021 Oct 14;13(1):153.

25. Engelen M, Kemp S, de Visser M, van Geel BM, Wanders RJA, Aubourg P, et al. X-linked adrenoleukodystrophy (X-ALD): clinical presentation and guidelines for diagnosis, follow-up and management. Orphanet J Rare Dis. 2012 Aug 13;7:51.

26. Cappa M, Todisco T, Bizzarri C. X-linked adrenoleukodystrophy and primary adrenal insufficiency. Front Endocrinol. 2023;14:1309053.

27. Berger J, Forss-Petter S, Eichler FS. Pathophysiology of X-linked adrenoleukodystrophy. Biochimie. 2014 Mar;98(100):135–42.

28. Kemp S, Berger J, Aubourg P. X-linked adrenoleukodystrophy: clinical, metabolic, genetic and pathophysiological aspects. Biochim Biophys Acta. 2012 Sep;1822(9):1465–74.

29. Wang Y, Busin R, Reeves C, Bezman L, Raymond G, Toomer CJ, et al. X-linked adrenoleukodystrophy: ABCD1 de novo mutations and mosaicism. Mol Genet Metab. 2011;104(1–2):160–6.

30. Wiesinger C, Eichler FS, Berger J. The genetic landscape of X-linked adrenoleukodystrophy: inheritance, mutations, modifier genes, and diagnosis. Appl Clin Genet. 2015;8:109–21.

31. Verrilli F, Kebriaei H, Glielmo L, Corless M, Vecchio CD. Effects of selection and mutation on epidemiology of X-linked genetic diseases. Math Biosci Eng MBE. 2017 Jun 1;14(3):755–75.

32. Vlachou S, Kanakis G, Kaltsas G. Adrenal Insufficiency Due to X-Linked Adrenoleukodystrophy. In: Feingold KR, Anawalt B, Blackman MR, Boyce A, Chrousos G, Corpas E, et al., editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000 [cited 2024 Sep 8]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK278944/

33. Berger J, Gärtner J. X-linked adrenoleukodystrophy: clinical, biochemical and pathogenetic aspects. Biochim Biophys Acta. 2006 Dec;1763(12):1721–32.

34. Mosser J, Lutz Y, Stoeckel ME, Sarde CO, Kretz C, Douar AM, et al. The gene responsible for adrenoleukodystrophy encodes a peroxisomal membrane protein. Hum Mol Genet. 1994 Feb;3(2):265–71.

35. Wiesinger C, Kunze M, Regelsberger G, Forss-Petter S, Berger J. Impaired very long-chain acyl-CoA β-oxidation in human X-linked adrenoleukodystrophy fibroblasts is a direct consequence of ABCD1 transporter dysfunction. J Biol Chem. 2013 Jun 28;288(26):19269–79.

36. Kemp S, Valianpour F, Mooyer PAW, Kulik W, Wanders RJA. Method for measurement of peroxisomal very-long-chain fatty acid beta-oxidation in human skin fibroblasts using stable-isotope-labeled tetracosanoic acid. Clin Chem. 2004 Oct;50(10):1824–6.

37. Engelen M, Kemp S, Poll-The BT. X-linked adrenoleukodystrophy: pathogenesis and treatment. Curr Neurol Neurosci Rep. 2014 Oct;14(10):486.

38. de Vasconcelos P, Lacerda JF. Hematopoietic Stem Cell Transplantation for Neurological Disorders: A Focus on Inborn Errors of Metabolism. Front Cell Neurosci. 2022;16:895511.

39. Lund TC, Stadem PS, Panoskaltsis-Mortari A, Raymond G, Miller WP, Tolar J, et al. Elevated cerebral spinal fluid cytokine levels in boys with cerebral adrenoleukodystrophy correlates with MRI severity. PloS One. 2012;7(2):e32218.

40. López-Erauskin J, Galino J, Ruiz M, Cuezva JM, Fabregat I, Cacabelos D, et al. Impaired mitochondrial oxidative phosphorylation in the peroxisomal disease X-linked adrenoleukodystrophy. Hum Mol Genet. 2013 Aug 15;22(16):3296–305.

41. Weinhofer I, Zierfuss B, Hametner S, Wagner M, Popitsch N, Machacek C, et al. Impaired plasticity of macrophages in X-linked adrenoleukodystrophy. Brain J Neurol. 2018 Aug 1;141(8):2329–42.

42. Bergner CG, van der Meer F, Winkler A, Wrzos C, Türkmen M, Valizada E, et al. Microglia damage precedes major myelin breakdown in X-linked adrenoleukodystrophy and metachromatic leukodystrophy. Glia. 2019 Jun;67(6):1196–209.

43. Ferrer I, Aubourg P, Pujol A. General aspects and neuropathology of X-linked adrenoleukodystrophy. Brain Pathol Zurich Switz. 2010 Jul;20(4):817–30.

44. Zhu J, Eichler F, Biffi A, Duncan CN, Williams DA, Majzoub JA. The Changing Face of Adrenoleukodystrophy. Endocr Rev. 2020 Aug 1;41(4):577–93.

45. Engelen M, van Ballegoij WJC, Mallack EJ, Van Haren KP, Köhler W, Salsano E, et al. International Recommendations for the Diagnosis and Management of Patients With Adrenoleukodystrophy: A Consensus-Based Approach. Neurology. 2022 Nov 22;99(21):940–51.

46. Parasar P, Kaur N, Singh J. Pathophysiology of X-Linked Adrenoleukodystrophy: Updates on Molecular Mechanisms. J Biotechnol Biomed. 2024;7(2):277–88.

47. Palakuzhiyil SV, Christopher R, Chandra SR. Deciphering the modifiers for phenotypic variability of X-linked adrenoleukodystrophy. World J Biol Chem. 2020 Nov 27;11(3):99–111.

48. Di Rocco M, Doria-Lamba L, Caruso U. Monozygotic twins with X-linked adrenoleukodystrophy and different phenotypes. Ann Neurol. 2001 Sep;50(3):424.

49. Sobue G, Ueno-Natsukari I, Okamoto H, Connell TA, Aizawa I, Mizoguchi K, et al. Phenotypic heterogeneity of an adult form of adrenoleukodystrophy in monozygotic twins.

Ann Neurol. 1994 Dec;36(6):912–5.

50. Korenke GC, Fuchs S, Krasemann E, Doerr HG, Wilichowski E, Hunneman DH, et al. Cerebral adrenoleukodystrophy (ALD) in only one of monozygotic twins with an identical ALD genotype. Ann Neurol. 1996 Aug;40(2):254–7.

51. Bonaventura E, Alberti L, Lucchi S, Cappelletti L, Fazzone S, Cattaneo E, et al. Newborn screening for X-linked adrenoleukodystrophy in Italy: Diagnostic algorithm and disease monitoring. Front Neurol. 2022;13:1072256.

52. Assies J, Gooren LJ, Van Geel B, Barth PG. Signs of testicular insufficiency in adrenomyeloneuropathy and neurologically asymptomatic X-linked adrenoleukodystrophy: a retrospective study. Int J Androl. 1997 Oct;20(5):315–21.

53. Lauer A, Speroni SL, Choi M, Da X, Duncan C, McCarthy S, et al. Hematopoietic stem-cell gene therapy is associated with restored white matter microvascular function in cerebral adrenoleukodystrophy. Nat Commun. 2023 Apr 5;14(1):1900.

54. van Haren K, Engelen M, Wolf N. Measuring early lesion growth in boys with cerebral demyelinating adrenoleukodystrophy. Neurology. 2019 Apr 9;92(15):691–3.

55. Mallack EJ, van de Stadt S, Caruso PA, Musolino PL, Sadjadi R, Engelen M, et al. Clinical and radiographic course of arrested cerebral adrenoleukodystrophy. Neurology. 2020 Jun 16;94(24):e2499–507.

56. Korenke GC, Pouwels PJ, Frahm J, Hunneman DH, Stoeckler S, Krasemann E, et al. Arrested cerebral adrenoleukodystrophy: a clinical and proton magnetic resonance spectroscopy study in three patients. Pediatr Neurol. 1996 Sep;15(2):103–7.

57. Huffnagel IC, van Ballegoij WJC, van Geel BM, Vos JMBW, Kemp S, Engelen M. Progression of myelopathy in males with adrenoleukodystrophy: towards clinical trial readiness. Brain J Neurol. 2019 Feb 1;142(2):334–43.

58. Manor J, Chung H, Bhagwat PK, Wangler MF. ABCD1 and X-linked adrenoleukodystrophy: A disease with a markedly variable phenotype showing conserved neurobiology in animal models. J Neurosci Res. 2021 Dec;99(12):3170–81.

59. Gujral J, Sethuram S. An update on the diagnosis and treatment of adrenoleukodystrophy. Curr Opin Endocrinol Diabetes Obes. 2023 Feb 1;30(1):44–51.

60. Schirinzi T, Vasco G, Aiello C, Rizzo C, Sancesario A, Romano A, et al. Natural history of a cohort of ABCD1 variant female carriers. Eur J Neurol. 2019 Feb;26(2):326–32.

61. Maier EM, Kammerer S, Muntau AC, Wichers M, Braun A, Roscher AA. Symptoms in carriers of adrenoleukodystrophy relate to skewed X inactivation. Ann Neurol. 2002 Nov;52(5):683–8.

62. Watkiss E, Webb T, Bundey S. Is skewed X inactivation responsible for symptoms in female carriers for adrenoleucodystrophy? J Med Genet. 1993 Aug;30(8):651–4.

63. Salsano E, Tabano S, Sirchia SM, Colapietro P, Castellotti B, Gellera C, et al. Preferential expression of mutant ABCD1 allele is common in adrenoleukodystrophy female carriers but unrelated to clinical symptoms. Orphanet J Rare Dis. 2012 Jan 26;7:10.

64. Hershkovitz E, Narkis G, Shorer Z, Moser AB, Watkins PA, Moser HW, et al. Cerebral X-linked adrenoleukodystrophy in a girl with Xq27-Ter deletion. Ann Neurol. 2002 Aug;52(2):234–7.

65. Huffnagel IC, Laheji FK, Aziz-Bose R, Tritos NA, Marino R, Linthorst GE, et al. The Natural History of Adrenal Insufficiency in X-Linked Adrenoleukodystrophy: An International Collaboration. J Clin Endocrinol Metab. 2019 Jan 1;104(1):118–26.

66. Engelen M, Barbier M, Dijkstra IME, Schür R, de Bie RMA, Verhamme C, et al. X-linked adrenoleukodystrophy in women: a cross-sectional cohort study. Brain J Neurol. 2014 Mar;137(Pt 3):693–706.

67. Turro E, Astle WJ, Megy K, Gräf S, Greene D, Shamardina O, et al. Whole-genome sequencing of patients with rare diseases in a national health system. Nature. 2020 Jul;583(7814):96–102.

68. Souche E, Beltran S, Brosens E, Belmont JW, Fossum M, Riess O, et al. Recommendations for whole genome sequencing in diagnostics for rare diseases. Eur J Hum Genet EJHG. 2022 Sep;30(9):1017–21.

69. Austin-Tse CA, Jobanputra V, Perry DL, Bick D, Taft RJ, Venner E, et al. Best practices for the interpretation and reporting of clinical whole genome sequencing. NPJ Genomic Med. 2022 Apr 8;7(1):27.

70. Clark MM, Stark Z, Farnaes L, Tan TY, White SM, Dimmock D, et al. Meta-analysis of the diagnostic and clinical utility of genome and exome sequencing and chromosomal microarray in children with suspected genetic diseases. NPJ Genomic Med. 2018;3:16.

71. Wright CF, Campbell P, Eberhardt RY, Aitken S, Perrett D, Brent S, et al. Genomic Diagnosis of Rare Pediatric Disease in the United Kingdom and Ireland. N Engl J Med. 2023 Apr 27;388(17):1559–71.

72. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med Off J Am Coll Med Genet. 2015 May;17(5):405–24.

73. Morris HR, Houlden H, Polke J. Whole-genome sequencing. Pract Neurol. 2021 May

10;21(4):322–7.

74. 100,000 Genomes Project Pilot Investigators, Smedley D, Smith KR, Martin A, Thomas EA, McDonagh EM, et al. 100,000 Genomes Pilot on Rare-Disease Diagnosis in Health Care - Preliminary Report. N Engl J Med. 2021 Nov 11;385(20):1868–80.

75. Sanford Kobayashi E, Waldman B, Engorn BM, Perofsky K, Allred E, Briggs B, et al. Cost Efficacy of Rapid Whole Genome Sequencing in the Pediatric Intensive Care Unit. Front Pediatr. 2021;9:809536.

76. Lowther C, Valkanas E, Giordano JL, Wang HZ, Currall BB, O'Keefe K, et al. Systematic evaluation of genome sequencing for the diagnostic assessment of autism spectrum disorder and fetal structural anomalies. Am J Hum Genet. 2023 Sep 7;110(9):1454–69.

77. Lindstrand A, Eisfeldt J, Pettersson M, Carvalho CMB, Kvarnung M, Grigelioniene G, et al. From cytogenetics to cytogenomics: whole-genome sequencing as a first-line test comprehensively captures the diverse spectrum of disease-causing genetic variation underlying intellectual disability. Genome Med. 2019 Nov 7;11(1):68.

78. Chen HA, Hsu RH, Chen PW, Lee NC, Chiu PC, Hwu WL, et al. High incidence of null variants identified from newborn screening of X-linked adrenoleukodystrophy in Taiwan. Mol Genet Metab Rep. 2022 Sep;32:100902.

79. Albersen M, van der Beek SL, Dijkstra IME, Alders M, Barendsen RW, Bliek J, et al. Sex-specific newborn screening for X-linked adrenoleukodystrophy. J Inherit Metab Dis. 2023 Jan;46(1):116–28.

80. Shimozawa N, Takashima S, Kawai H, Kubota K, Sasai H, Orii K, et al. Advanced Diagnostic System and Introduction of Newborn Screening of Adrenoleukodystrophy and Peroxisomal Disorders in Japan. Int J Neonatal Screen. 2021 Aug 25;7(3):58.

81. Ding Y, Owen M, Le J, Batalov S, Chau K, Kwon YH, et al. Scalable, high quality, whole genome sequencing from archived, newborn, dried blood spots. NPJ Genomic Med. 2023 Feb 14;8(1):5.

82. Wang X, Sun Y, Guan XW, Wang YY, Hong DY, Zhang ZL, et al. Newborn genetic screening is highly effective for high-risk infants: A single-centre study in China. J Glob Health. 2023 Oct 13;13:04128.

83. Minskaia E, Galieva A, Egorov AD, Ivanov R, Karabelsky A. Viral Vectors in Gene Replacement Therapy. Biochem Biokhimiia. 2023 Dec;88(12):2157–78.

13. BIOGRAPHY

I was born in Mississauga, Ontario, Canada, where I attended elementary and high school. I earned a bachelor's degree in medical sciences from Western University. During my undergraduate studies, I actively participated in HOSA - Future Health Professionals, where I served as a trainer preparing students for competition in the Forensic Science category. Following graduation, I enrolled at the School of Medicine, University of Zagreb. During my time as a medical student, I was awarded the Certificate of Excellence in Pathophysiology in the academic year 2020/2021 and the Dean's Award for academic excellence in 2022/2023. I served as an elected class representative and was an active member of the 2018/2019 and 2023/2024 eMed Student Council. I have been awarded the STEM scholarship, issued by the Ministry of Science and Education, and the Scholarship for Academic Excellence, issued by the City of Zagreb. I am part of the CROseq-GenomeBank Project, which is the first Croatian Genome Aggregated Database (CGAD; *Genoma*) and the first Croatian Genome-Phenotype Database (CGPD; *FeGena*), aimed at aiding the diagnosis of rare, genetic conditions in the pediatric population in Croatia.