Clinical and molecular insights into BAFopathies

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Chapter 1

Introduction

INTRODUCTION

BAFopathies are disorders caused by pathogenic germline variants in genes encoding part of the BAF (BRG_1/BRM associated factor) chromatin remodeling complex (Figure 1). Altogether, BAFopathies are one of the main genetic causes of intellectual disability (ID)[1,2]. Based on the phenotype and molecular cause, BAFopathies can be divided into different entities, of which Coffin-Siris syndrome (CSS, OMIM#135900) is the most frequent.

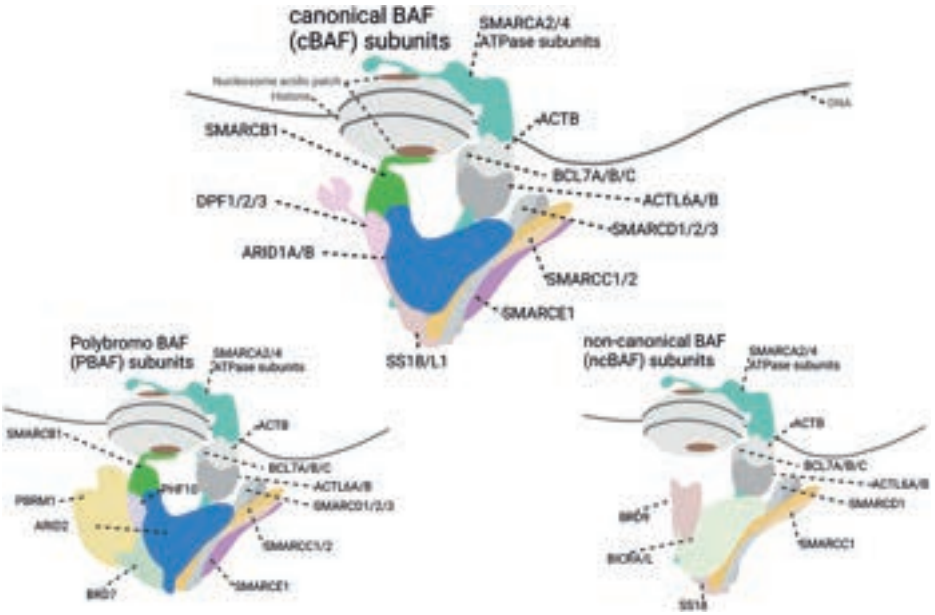

Figure 1: BAF-complex.
BAF-complex structure is derived from Mashtalir et al. 2021 [53], Wei et al 2023 [54], and Valencia et al 2023[1].

Coffin-Siris syndrome

CSS was first described in 1970 by dr. Coffin, a pediatrician, and dr. Siris, a radiologist[3]. Originally, CSS (also known as '5th digit syndrome') was diagnosed based on clinical criteria, including a short 5th finger, 5th finger or toenail hypoplasia, coarse facial features, hypertrichosis and intellectual disability or developmental delay frequently coinciding with a range of other features[4,5].

The causative genes of CSS remained unknown until next generation sequencing (NGS) in 2012 allowed the identification of the first genes causing CSS (i.e. *ARID1B*, *ARID1A*,

SMARCA4, *SMARCB1* and *SMARCE1*)[6,7] in patients with a clinical diagnosis of CSS (Figure 2). The publication describing *ARID1B* as a cause of CSS also identified a number of patients with a deletion of *ARID1B*, some without the typical features of CSS, indicating that perhaps *ARID1B* could be an important cause of ID[6]. This was confirmed around the same time by another publication identifying pathogenic variants in *ARID1B* as a frequent cause of ID[8]. Later, large-scale NGS studies in less selected patient groups (i.e. ID, neurodevelopmental delay (NDD), autism) confirmed that *ARID1B* is one of the most frequent causes of ID at about 1%[2,9-13]. Given the often-subtle features of CSS it is perhaps not surprising that the diagnosis of CSS has since then transitioned from a clinical diagnosis to one more and more based on genotype.

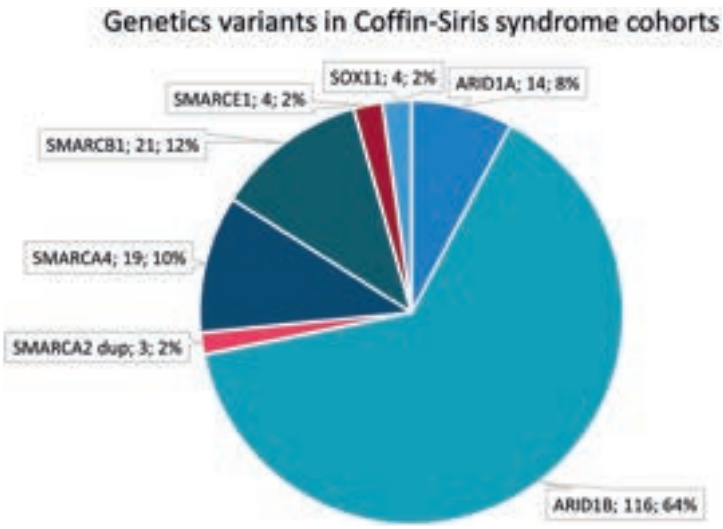

Figure 2: CSS genes pie chart based on reported CSS cohorts [7,55-58]*
**PHF6* and *SMARCA2* variants were also identified in a number of clinical CSS patients, in retrospect these patients were diagnosed as having Börjeson-Forssman-Lehmann syndrome (*PHF6*) and Nicolaides-Baraitser syndrome (*SMARCA2*).

Other BAFopathies

Aside from CSS, several other syndromic causes of ID have been linked to the BAF-complex. Nicolaides Baraitser syndrome (NCBRS, OMIM 601358) has been associated with pathogenic missense variants in *SMARCA2*[14], while whole gene duplications of *SMARCA2* have been linked to a CSS-like phenotype[15]. Other BAF-complex genes linked to a CSS-like phenotype are *ARID2*[16], *BICRA*[20], *DPF2*[17], *SMARCC2*[18], *SMARCD1*[27] and, *SOX11*[19]. Furthermore, pathogenic variants in *ACTL6A*[21], *ACTL6B*[22,23] and, *SMARCC1*[24-26] have each been associated with a gene-specific (syndromic) ID or NDD phenotype. As illustrated in Figure 1 all these genes encode parts of the BAF-complex except *SOX11*.

SOX11 is a downstream transcriptional factor of the BAF complex[19,28]. Interestingly, specific pathogenic variants in a number of the CSS and NCBRS genes lead to other non-CSS phenotypes. A phenotype of severe ID and choroid plexus hyperplasia has been observed in patients with a specific *SMARCB1* variant p.(Arg37His)[29], *SMARCA2* variants outside the helicase domains of *SMARCA2* have been observed in patients with blepharophimosis intellectual disability syndrome[30] (BIS, OMIM 619293) and, whole gene duplications of *ARID1A* cause a distinct syndrome ID[31] (Figure 3). All of this illustrates the broad phenotypic spectrum associated with these BAFopathies.

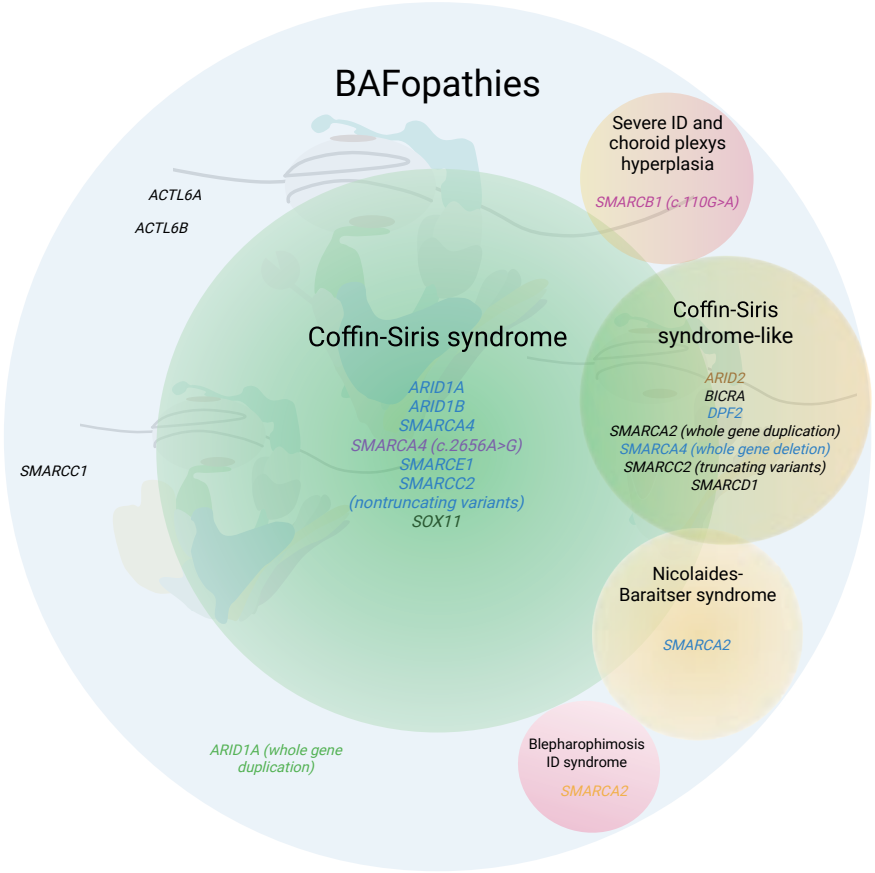

Figure 3: Genetic and epigenetic landscape of BAFopathies.
BAFopathies, caused by pathogenic variants in genes encoding components of the BAF complex, can be categorized into distinct entities based on genotype and phenotype. Disorders with specific names are written in regular font, while gene names are italicized. Additionally, colors indicate the episignature associated with pathogenic variants in these genes, where applicable. The size of the circles approach the number of patient in these groups, but are not an exact estimate.

BAF-complex

The genes mutated within BAFopathies all encode subunits of the BAF-complex or are a transcription factor downstream of BAF-complex (e.g. *SOX11*). As illustrated by Figure 1, there are three different configurations of the BAF-complex called canonical BAF-complex, noncanonical BAF-complex and polybromo BAF-complex. These distinct subunit assemblies of the BAF-complex occur at different time points during development and in different tissues, indicating specific roles of each subunit.

Current issues in BAFopathies

Over the past decade the application of NGS has brought us the identification of BAFopathies and enabled the discovery, diagnosis and description of several gene-specific phenotypes. However, this development has also revealed several challenges concerning 1) the interpretation of genetic variants, 2) shortage of genotype-phenotype studies and, 3) the lack of available treatments for the increasing number of diagnosed patients with BAFopathies.

1) Variant interpretation

The increasing application of NGS in genetic diagnostics has led to the identification of many variants in BAFopathy genes. Some of these variants are clearly pathogenic, but an increasing number is considered a variant of uncertain significance (VUS). Interpretation of these VUS is particularly challenging because of the broad, and often not very specific, phenotype associated with BAFopathies.

To aid and standardize the interpretation of genetic variants the American College of Medical Genetics and Genomics (ACMG) formulated a framework[32]. The ACMG framework can be used to interpret the variant into five classes: benign, likely benign, VUS, likely pathogenic, and pathogenic. Important aspects in the ACMG framework in the context of BAFopathies are whether the variant occurs in control populations, whether the variant segregates with the disease or is *de novo*; and whether the phenotype fits the known phenotype associated with the gene.

Whilst the ACMG criteria generally serve their purpose, they are formulated in a very general manner to apply to all genes and genetic variants. However, there are instances where these criteria may prove insufficient, necessitating (gene-specific) amendments[33-37]. For example, based on the ACMG framework a missense variant in a known ID-gene could be regarded likely pathogenic when it occurs *de novo* and absent in control populations. The UK Association for Clinical Genomic Science (UK-ACGS) published a specification for variant interpretation in which they specified that in the context of a high penetrant monogenic disorder a *de novo* ACMG-criterium (PS2)

should only be applied if the patient's phenotype is consistent with the gene-phenotype association.

Phenotype assessment

Classically, in the context of ID syndromes the phenotype has always been a key determinant in classifying a VUS. In this assessment it may be very helpful to consider whether the clinician suspected this gene or condition before genetic testing. This is to avoid a Texas sharpshooter fallacy, where differences are ignored and similarities overemphasized, like a sharpshooter drawing targets around random bullet holes. Just because certain clinical characteristics match those commonly associated with a gene, this does not necessarily make the characteristics specific for the disorder linked to that gene. Similar features could manifest in patients with other conditions. The assessment of whether a phenotype is fitting for a gene depends on human interpretation and is therefore subjective. A more objective approach to assessing the phenotype is through Human Phenotype Ontology (HPO) terms[38].

HPO-terms and PhenoScore

HPO is a standardized vocabulary of phenotypic abnormalities encountered in human disease. Each term represents a phenotypic abnormality. All terms are connected via a tree-like structure in a class-subclass relationship. For example, the HPO-term 'intellectual disability (HP:0001249)' is a subclass of 'Neurodevelopmental abnormality (HP:0012759)'. HPO-terms can be used in interpretation pipelines for genetic testing. Genes associated with the phenotype of the patient can be prioritized using HPO-terms.

HPO-terms can also be used in deep phenotyping. Deep phenotyping is the precise and comprehensive analysis of phenotypic abnormalities. An example of deep phenotyping via HPO-terms is PhenoScore[39]. PhenoScore is an artificial intelligence-based phenomics framework, which integrates facial recognition technology and HPO data analysis to quantify phenotypic similarity. PhenoScore can assess, based on HPO-terms and facial photographs, whether a patient group can be distinguished from matched controls. Consequently, PhenoScore can be used in VUS interpretation. PhenoScore can calculate a numerical score representing the similarity between a patient's phenotype and the expected phenotype of the disorder. In this manner PhenoScore provides an objective measure of phenotypic similarity, can identify group specific features, and aid in VUS interpretation. For example, the PhenoScore clustering result of a *de novo* VUS could be used to determine whether a *de novo* criterium (PS2) and specific phenotype criterium (PP4) may be applied.

If a VUS has been identified, assessing whether a patient's phenotype fits the expected phenotype of the VUS-gene is especially complicated in the context of BAFopathies. This is because there have already been several instances described where specific variants in a single gene lead to different syndromes. Examples are that (1) deletion and truncating variants in *ARID1A* lead to Coffin-Siris syndrome[7], but whole gene duplications lead to a different ID-phenotype[31], or, (2) missense variants in *SMARCB1* C-terminal domain lead to CSS[7], while one specific missense variant in the winged helix domain (near the N-terminal) leads to a specific non-CSS phenotype[29]. Deep phenotyping, extensive clinical descriptions and PhenoScore analyses could assist interpretation in BAFopathies.

Other tools of variant classification

There are several tools that could provide insight into the potential pathogenicity of a VUS. For example, functional readouts—such as introducing the variant into cellular or animal models—can help clarify the biological impact of the variant. These approaches are usually time-consuming, expensive and require the presence of an existing disease model.

DNA methylation

Another approach to aid variant interpretation, requiring only the leftover DNA from genetic testing is the analysis of DNA methylation patterns. DNA methylation is essential for gene regulation. DNA methylation patterns differ between tissue types[40], and can also differ between patients and controls. Specific DNA methylation patterns in blood have been linked to several disorders, such as cancer, neurodevelopmental disorders[41], and genetic syndromes[42,43], such as BAFopathies[44] (Figure 3). These methylation patterns can be used to define patient groups and can afterwards be used for VUS interpretation. For example, pathogenic variants in CSS and NCBRS genes lead to a BAFopathy episignature in blood. Several patients with a missense variant in *SMARCA2* and an ID phenotype, but not specific for NCBRS did not have this BAFopathy episignature in their blood, but were later found to have a different BIS-specific episignature.

2) Genotype-phenotype studies and their potential biases

A second issue for BAFopathies is the lack of genotype-phenotype studies. A genetic diagnosis, based on a (likely) pathogenic variant in a BAFopathy gene enables the clinician to counsel parents about the (expected) phenotype and give screening recommendations. This information is based on genotype-phenotype studies of the diagnosed BAFopathy. Genotype-phenotype studies asses clinical phenotypes of patients and divide this clinical description in gene and, if applicable, genotype

specific groups. Since genotype-phenotype studies serve as the information source for counseling patients, parents, and caregivers, it is essential to acknowledge potential pitfalls and biases inherent in such studies. For example, in the case of CSS, it is important that phenotypes are assessed per gene because there are important differences between patients with pathogenic variants in different CSS genes, and even among patients harboring pathogenic variants in the same gene. Aside from this, there are several potential biases inherent in genotype-phenotype studies.

Ascertainment bias is such an example. Patients diagnosed after a suspicion of a specific syndrome may have a different phenotypic spectrum than a patient diagnosed after whole exome or genome sequencing. Patients with a pre-test suspicion may be more likely to have the syndrome specific features compared to patients identified to genome-wide sequencing ID without any suggestive features. Research examining potential differences among these patient groups is therefore essential.

Another type of bias is phenotype reporting bias. In larger cohort studies, the presence or absence of certain features may be unknown in a subset of patients. Excluding these patients from prevalence estimation can lead to overestimation, while counting them as lacking the feature may result in underestimation. To address this type of bias, it is advisable to report a prevalence range for such features or use data collection techniques that allow annotation of the unknown status, providing a more accurate representation.

Yet another type of bias is associated with the age of patients at inclusion. Due to the advancements in NGS children are predominantly diagnosed with rare disorders, leading to an underrepresentation of adult-aged patients in reported cohorts. This discrepancy can affect the evaluation of age-related skills or features, such as seizures or other potential adult-onset manifestations. It is therefore valuable to analyze developmental features, like the age of first words, in survival analyses and separately examine adult-aged patients to offer a more comprehensive overview of features associated with older age. This ensures a more nuanced understanding of age-related characteristics of the studied disorder over time.

3) Etiology and treatment of BAFopathies

To enhance counseling, interpretation of a genetic variant, obtaining a genetic diagnosis and genotype-phenotype studies are paramount, but ongoing research into the pathophysiology of BAFopathies is also necessary for the improvement of counseling and care. Therefore, the third challenge within BAFopathies is the lack of potential treatment options. To propose viable treatment options understanding of the etiology

is needed. Exploring the underlying pathophysiology of BAFopathies through disease models, such as animal (*in vivo*) models and patient-derived cell (*in vitro*) models or organoids is valuable because these models do not only deepen our understanding of the disorder but also pave the way for potential disease-specific therapeutic interventions.

BAFopathy-based animal models, such a Arid1b-haploinsufficient mice[45], can be used to study disease mechanisms. An advantage of an animal model is that disease progression can be studied in a living organism, an organism with a shorter lifespan and (brain) tissue samples can be taken. And, after a clear phenotype is established, therapeutic interventions can be tested. An example is described by Jung et al[46]. In their Arid1b-haploinsufficient mouse model they identified an increased apoptosis and decreased proliferation of inhibitory GABAergic interneurons. Consequently, they hypothesized and demonstrated that clonazepam through enhancing the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) may rescue the mice's phenotype. A disadvantage of animal models is the question whether findings from animal models can be extrapolated to humans. A possible next step could be validating the finding in human cell models[47-50] or organoids[51,52].

Patient-derived cell models and organoids have the advantage of investigating the effect of a variant in the genetic background of the individual. Although in extrapolating the findings to other patients this could be a disadvantage and iso-genic cell lines (e.g. CRISPR-Cas) may be preferred. Cell models can be used to examine (aberrant) DNA methylation, gene expression pattern via RNA-seq and investigate dysregulated pathways.

These models can be used not only to investigate the pathophysiology of genetic variants but also to explore potential treatment effects. Proposing and testing a potential disease-specific therapy within a disease model is an essential step in therapy development, before efficacy can be investigated in patients. In rare disorders like ID, limited patient numbers and heterogeneity within the disorder pose challenges to designing effective interventions. Publication of research into potential therapeutic approaches for rare ID disorders not only benefits specific patient groups but also serves as a valuable example for future therapeutic studies involving ID patients.

Aim and scope of this thesis

All of the above illustrates the intricacies of variant interpretation, the importance of genotype-phenotype studies, and the need for etiological and treatment studies in BAFopathies. To address these issues the research in this thesis aims 1) to provide

further insight in the clinical and epigenetic phenotypes associated with pathogenic variants in BAFopathy genes; and; 2) further examine the methods for studying mechanisms of disease and possible treatment by creating a model to further study possible underlying etiological aspects and by studying the potential effect of clonazepam treatment in *ARID1B* patients.

Outline of this thesis

1) Insights into clinical and epigenetic phenotypes

In **chapter 2** we describe 143 patients with pathogenic variants in *ARID1B* to answer the question whether there is a difference between patient diagnosed with pathogenic variant after a suspicion of CSS and patients diagnose after whole exome or genome sequencing. In this chapter we broaden the phenotypic spectrum associated with pathogenic *ARID1B* variants, we investigate the effects of ascertainment bias and phenotype reporting bias in our cohort and examine different data collection methods. In **chapter 3** we investigate the fetal CSS phenotype by describing prenatal anomalies detected among patients with pathogenic variants in CSS-associated genes, highlighting a new part of the phenotype associated with CSS, and providing an explanation why variants in *ARID1A* are so much rarer than variants in *ARID1B*.

A subset of CSS patients have a distinct DNA methylation pattern present in their blood, also referred to as the BAFopathy episignature[44]. This episignature provides a valuable tool in diagnostic care in VUS interpretation. In **chapter 4** we use this episignature, together with the predecessor of the PhenoScore algorithm, to interpret inherited *ARID1B* variants and evaluate the ACMG criteria in the context of *ARID1B*. Our analysis sheds light on the challenges associated with interpreting inherited *ARID1B* variants and provides recommendations concerning the ACMG-criteria to aid the interpretation of such variants. In **chapter 5** we address age-dependent bias by describing 87 adult-aged patients with pathogenic variants in *ARID1B* to give insight in the development of these patients and any features that develop with age. Based on this information we formulate screening recommendations.

In **chapter 6**, where we report the *ARID1B* microduplication syndrome and compare the DNA methylation pattern and phenotype of patients with a whole gene duplication with data of patients with an *ARID1A* duplication.

2) Methods for studying mechanisms of disease and possible treatment

In **chapter 7** we describe an *in vitro* model system to study CSS variant effects in neuronal differentiation of induced Pluripotent Stem Cells (iPSC) of patients with pathogenic variants in *ARID1B* and *SMARBC1*. These iPSCs are differentiated towards

GABAergic interneurons to investigate DNA methylation throughout neuronal development and whether the increased apoptosis observed in Arid1b haploinsufficient mice[46] also occurs in human cells.

In this same mouse model[46], these researchers hypothesized and demonstrated that due to the reduced number of inhibitory GABAergic interneurons, administering clonazepam, which enhances the activity of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), partially rescues the phenotype of Arid1b haploinsufficient mice. In **chapter 8** we investigate via a randomized, double-blind, crossover study followed by an n-of-1 design whether clonazepam administration has a similar effect in *ARID1B* patients.

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