ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

DOTTORATO DI RICERCA IN

SCIENZE MEDICHE GENERALI E SCIENZE DEI SERVIZI

Ciclo 37

Settore Concorsuale: 06/G1 - PEDIATRIA GENERALE, SPECIALISTICA E NEUROPSICHIATRIA INFANTILE

Settore Scientifico Disciplinare: MED/38 - PEDIATRIA GENERALE E SPECIALISTICA

BIOLOGICAL BASIS OF INTELLECTUAL DISABILITY IN CHILDREN WITH DOWN SYNDROME

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Esame finale anno 2025

Abstract

Down syndrome (DS), the most common known genetic cause of intellectual disability (ID), is marked by specific areas of strength and challenge in relation to developmental level. In his studies, Lejeune hypothesized that DS could be considered a metabolic disease and this hypothesis was confirmed by different studies in literature. In particular, one-carbon metabolism was considered to be imbalanced in subjects with DS.
Considering the presence of numerous studies that link the one-carbon cycle with central nervous system development we tried:
- to increase knowledge about the cognitive profile of children with trisomy 21, understanding strengths, weaknesses and developmental trajectories in order to identify aspects on which to act;
- to develop a machine learning approach capable of analysing large datasets containing clinical, metabolic, laboratory, and genetic data in order to analyse such a complex aspect as cognition;
- to further our knowledge of metabolites associated with the one-carbon cycle, in particular with metabolites that have not yet been studied (eg. THF, 5-m-THF, 5-f-THF, SAM and SAH), in patients with trisomy 21 and to study possible associations between them and cognitive aspects.
All participants were recruited during the annual follow-up of the individuals with DS at the Day Hospital of the Neonatology Unit, IRCCS Sant'Orsola-Malpighi Polyclinic, Bologna, Italy. Children and adolescents were assessed at the Department of Developmental Psychology at the University of Padova. Metabolic data were analysed in genomic laboratory of university of Bologna.
We were able to highlight the presence of 3 different cognitive profiles and confirmed, among the executive functions, which represent strengths and weaknesses. We have seen, moreover, how executive functions could influence the development of adaptive skills and that while children with DS continue to acquire adaptive skills over time, the gap with typically developing peers widens, influenced by both cognitive level and environmental factors. We developed a machine learning algorithm that may be able to help in both research and clinical support by highlighting important cognitive developmental features to be taken into account. Moreover, our results confirm that there is a dysregulation of the one-carbon pathway in subjects with DS that could be related to cognitive impairment, in particular with executive functioning.

1. Introduction

Down syndrome (DS) was first described by John Langdon Down in 1866 (Down 1866) and later more precisely characterized by Jackson and Hall, who identified 10 (Hall 1966) or 25 (Jackson et al. 1976) key signs useful for clinical diagnosis. The relevance of these signs was reaffirmed by a study published by Locatelli and colleagues in 2022. One of the main aspects involved in this condition is wide spectrum of intellectual disability that affects all subjects with this condition and other clinical aspects frequently associated such as cardiovascular disease and hypothyroidism (Locatelli et al. 2022).

Only in 1959 a French paediatrician Prof. Jerome Lejeune associated this condition to the presence of a third copy of chromosome 21 (Hsa21) (Lejeune et al. 1959). Years later emerged clinical cases where copies of a limited segment of the said chromosome were associated with clinical manifestation of DS. This condition, called partial trisomy or incomplete trisomy, has been reported in about 200 cases and has led to the understanding that not all loci present on chromosome 21 are necessary for the manifestation of this syndrome (Pelleri et al. 2016). Analysing this condition, Pelleri and coll. in 2016 showed how the characteristic features of Down syndrome are related to a small region present on chromosome 21 (Highly restricted Down syndrome critical region, HR-DSCR) that spans only 34 kilobases (Pelleri et al. 2016). The HR-DSCR region has emerged as critical for the phenotype because it is always present in three copies in individuals diagnosed with DS and always present in two copies in individuals without a DS diagnosis as confirmed by other two papers in 2019 (Pelleri et al. 2019) and 2022 (Pelleri et al. 2022).

1.1 Cognitive Profile of subjects with Down syndrome

DS or Trisomy 21(T21), the most common known genetic cause of intellectual disability (ID), is marked by specific areas of strength and challenge in relation to developmental level (Will et al. 2021). Within-syndrome heterogeneity is also commonly observed across many dimensions as showed by a paper made by Onnivello and coll. In 2023 where they highlighted three developmental profiles in infant with DS (3.9–17.6 months). They found a ‘Mild Delay’ Profile, an ‘Moderate Delay’ Profile and a ‘Pronounced Delay’ Profile and showed that chronological age, having received heart surgery and having received occupational therapy could be associated with probability of profile membership.

In 2009, Tsao and Kindelberg explored the possible existence of different cognitive profiles in children with DS between 6 and 11 years old. Using a clustering approach they identified four separate cognitive profiles: the first cluster was characterized by relatively equivalent scores, close to the means, on both the verbal and nonverbal tests; the second group encountered difficulties in each subtest, but most obviously so in the verbal tests; the third profile was notable for its superior performances on the verbal subtests and the last group was characterized by its performances on the nonverbal tests.

Moreover, Tsao and Kindelberg found that performances on verbal and nonverbal tests did indeed improve during childhood between 6 and 10 years in the DS children while a stagnation, and even a slight regression can be observed between the ages of 10 and 11 on every subtest, except for practical adaptation (Tsao and Kindelberg 2009).

1.1.1 Language

Babies with Down syndrome (DS) shows difference in communication compared to the typically developing (TD) children the very early beginning of life.

Mundy and coll. (1995) found that children with DS showed lower rates of requesting, but not commenting, compared to TD language matched children. The development of vocalizations in infants with DS is generally similar to the development in TD infants (Dodd, 1972; Smith and Oller, 1981; Smith and Stoel-Gammon, 1983), although the onset of canonical babbling (i.e., adult- like consonant–vowel combinations) is delayed (Lynch et al. 1995). Some studies have found that gesture use is a strength in children with DS when compared to TD children (Roberts et al. 2007); the use of gestures in the early stages of communicative development is similar to TD infants while a greater use of gestures was related to better later language development for children with DS in two studies (Mundy et al. 1995; Yoder and Warren, 2004).

Focusing on phonology, differences in speech sound development for children with trisomy 21 begin to emerge with the transition to first words (Stray-Gunderson, 1986). Roberts and coll. (2005) found that preschool- and school-aged boys with DS experienced delays in speech development, exhibiting more sound errors, increased use of phonological processes, and distinct error patterns compared to younger typically developing boys matched for mental age on a single-word articulation test.

Considering semantics, the onset of the first spoken word is often delayed, and early expressive vocabulary growth is slow for children with DS (Caselli et al. 1998; Mervis and Robinson, 2000; Berglund et al. 2001). This can be associated with their deficits on measures of expressive

vocabulary, grammar and verbal short-term memory. In contrast, their receptive vocabulary skills are better developed and not significantly below the level expected for their nonverbal mental age. Children with DS have a broad language deficit that is shown in all language domains except for the receptive vocabulary (e.g. Chapman et al. 1991; Caselli et al. 1998; Chapman et al. 2000; Abbeduto et al. 2003; Laws & Bishop, 2003), they do not support claims that receptive language skills in general are better than expressive skills (Næss et al. 2011).

Compared with vocabulary, syntax is a particular weakness for individuals with trisomy 21. Children with DS are generally delayed in transitioning from 1- to 2-word speech (Iverson et al. 2003). After production of multiword speech has begun, children and adolescents with DS produce shorter utterances than younger TD nonverbal mental age matches (Miller, 1988; Rosin et al. 1988; Chapman et al. 1998). Despite these morphosyntactic weaknesses, adolescents do not reach a ‘‘syntactic ceiling’’ or stop growing in their syntax (Fowler, 1990; Fowler et al. 1994) but continue to advance in utterance length and syntax complexity through at least 20 years of age (Chapman et al. 2002; Thordardottir et al. 2002).

When focusing on conversation content, children with trisomy 21 often have poor speech intelligibility (Næss et al. 2011) and are less likely than typically developing (TD) children matched for mental age to consider their listener's needs, such as by signaling for clarification during communication breakdowns or using referential frames or scaffolding to aid listener comprehension (Abbeduto and Murphy, 2004; Abbeduto et al. 2006). Weaknesses in referential communication were linked to expressive language deficits in adolescents and young adults with DS (Abbeduto et al. 2006). However, adolescents with trisomy 21 demonstrate relative strengths in narrative skills when visual supports are provided. For instance, when telling stories with the aid of visual supports, such as a wordless picture book or film, adolescents with DS included more story elements than controls matched for syntax comprehension or production (Boudreau and Chapman, 2000; Miles and Chapman, 2002).

1.1.2 Visuo-spatial Abilities

Spatial abilities broadly refer to the capacity to understand and remember spatial relations. They help one to manipulate, construct and navigate the physical world (Newcombe & Shipley 2015). DS is characterized by relatively stronger visuo-spatial than verbal abilities (Meneghetti 2017) but spatial abilities are found to be particularly sensitive to ageing in adults with DS and may serve as early indicators of dementia for people with DS (Devenny et al. 2000; Sabbagh & Edgin 2015).

For characterizing spatial abilities Newcombe & Shipley in 2015 developed a theoretical framework based on empirical cognitive, neural and linguistic evidence double-dimension. This framework identified two spatial dimensions: 'static vs. dynamic', whether spatial skills are associated with movement, and 'extrinsic vs. intrinsic', whether the represented spatial relations are outside an object or within an object. These two dimensions were divided into four categories: static–extrinsic, static–intrinsic, dynamic–extrinsic and dynamic–intrinsic (Newcombe & Shipley 2015).

Static–extrinsic skills refer to encoding an object's spatial locations relative to other objects or an external reference frame and it corresponds to spatial perception (Linn & Petersen 1985). In this category, Morris and coll. (2023) have examined scaling and found no difference between participants with DS and their mental age (MA)-matched TD peers. Considering environmental information, asking participants to find the shortest route between two places individuals with DS made more mistakes in reproducing a path but most of them reached their destination (Meneghetti et al. 2017).

Static–intrinsic skills focus on spatial features of objects (e.g. size and configuration) and do not involve movement (Newcombe & Shipley 2015). It corresponds to closure speed, flexibility and spatial visualisation in traditional psychometric research categorising spatial abilities (e.g. Linn & Petersen 1985; Lohman et al. 1987; Carroll 1993). Even if there are some discordant studies in literature, most studies suggest that static–intrinsic skills may be an area of deficit for people with DS (Bracken et al. 2023).

Dynamic–extrinsic skills require transforming the interrelations of objects when the objects or the viewer move. Only one study has examined perspective taking in people with DS and found no difference between participants with DS and their MA-matched TD peers (Morris et al. 2023).

Dynamic–intrinsic skills involve transforming spatial relations, such as rotation, cross-sectioning and folding. Compared with other spatial categories, dynamic–intrinsic skills have received the most attention in DS research. It corresponds to mental rotation, spatial visualisation and spatial relations/speeded rotation in the traditional psychometric approach categorising spatial abilities. A thorough literature review (Yang et al. 2014) concluded that, for DS patients, block design performance may be fairly consistent with general cognitive ability while the evidence regarding mental rotation in people with DS is inconclusive at this stage (Bracken et al. 2023).

1.1.3 Motor Skills

The term motor skills refers to the ability to control and coordinate movements of the central nervous system and the muscles of the body and it is typically categorized into two main types: gross motor skills (GMS), which involve the use of larger muscle groups and are related to activities that require strength, balance and coordination of the whole body and fine motor skills (FMS), which involve the use of smaller muscle groups, particularly those in the hands and fingers, and are related to activities that require precise control and dexterity. Therefore, motor skills improve over time through practice and experience and are crucial for everyday tasks (Muñoz-Llerena et al. 2024) specifically, GMS support everyday movements and are linked to better overall health and development.

Children with T21 typically show lower GMS, especially in balance, impacting their safety and independence. Low muscle tone and less muscular tension force to exert more effort during activities that are needed to activate muscle mass causing a difficulty in maintaining postural stability and fatigue setting in almost immediately (Norton et al. 2016). Therefore, children with DS often display decreased muscle strength, decreased activity tolerance and endurance and a rounded shoulder posture (Norton et al. 2016). These factors hinder both GMS and FMS development, making it essential to apply targeted training.

Research indicates that balance is linked to motor skills, in particular an improved GMS is associated with better postural control. Children with DS often adopt inefficient strategies to maintain balance and causing a complication of their motor function. FMS, which often resulted more impaired in children with DS, require focused training on upper body strength and coordination (Muñoz-Llerena et al. 2024).

Considering data literature, strength training have large to moderate effects on general strength, moderate to small effects on maximal strength, and small effects on functional mobility tasks when neuromuscular training is used (Norton et al 2016). Protocols for strength and balance training should include horizontal jumps, vertical jumps, one-leg stances with eyes open, tandem stances, walking on lines, walking on balance beams, and jumping on a trampolines (Schalock et al 2012).

1.1.4 Memory

Memory includes three main domain divided as follows: Short-Term memory, Late-Term memory and Working memory.

Short-term memory (STM) refers to a limited capacity, immediate memory system in which small amounts of information can be actively upheld and preserved for a matter of seconds (Godfrey & Lee, 2018). One well-characterized cognitive challenge in DS is memory consolidation, with the STM component particularly affected throughout the lifespan, beginning in school age and into adulthood (Godfrey & Lee, 2018), indeed Children with DS have a weakness in digit and word recall tasks (Godfrey and Lee 2018), and this may influence their performance on the measures of grammatical ability (Næss et al. 2011). In comparison to TD youth, most studies suggest comparable or poorer performance also on nonverbal STM tasks (Lanfranchi et al. 2014) even if nonverbal STM skills are considered as a relative strength compared with verbal STM abilities within DS (Godfrey and Lee 2018). Conversely, visuospatial STM is considered a relative strength within DS. (Carney et al. 2013; Jarrold & Baddeley, 1997; Kogan et al. 2009) and visuospatial aids have also been found to improve performance on verbal STM tasks in children and adolescents with DS (Duarte et al. 2011).

Lower STM capacity has a cascading effect on working memory and long-term memory, as research shows that challenges with immediate memory prevent sufficient processing and long-term storage of information (Baddeley & Jarrold, 2007; Broadley et al. 1995; Cowan, 2008; Lanfranchi et al. 2004).

Long-term memory (LTM) refers to the process of storing information that can be accessed later in time. It involves three key stages: encoding, which extracts distinct elements to form a memory; storage, which maintains those memories; and retrieval, which involves accessing stored information (Eichenbaum, 2001).

Adolescents DS groups perform significantly worse on list-learning tasks with a delay and had significantly more intrusive responses and impaired discrimination abilities on list recall compared to MA-matched controls (Nichols 2004). This pattern is still confirmed on verbal long-term memory (LTM) assessments, visual associative memory tasks, pattern recognition, and spatial LTM tasks (Godfrey and Lee, 2018). Similarly to their verbal LTM abilities, most studies indicate that adolescents with DS show impaired performance on nonverbal LTM tasks when compared to MA-matched TD children (Godfrey and Lee, 2018). Adults with DS continue to perform below MA expectations on LTM tasks (Vicari et al. 2000) with nonverbal LTM memory that begins to decline in young adulthood (Crayton et al. 1998, Hon et al. 1998). Impairments are evident (in most studies) from an early age and persist across development (Godfrey and Lee 2018).

At least, working memory (WM) refers to the ability to maintain and manipulate information for a brief period of time. Similar to STM, WM involves temporary storage of limited amounts of information, but it also requires maintenance and attention while simultaneously processing information, avoiding distraction, and/or engaging in cognitive shifting (Alloway and Copello 2013).

In individuals with DS, difficulties with working memory have been linked to critical areas of development including academic achievement and adult employment (Will et al. 2017; Tomaszewski et al. 2018). Indeed, subjects with DS are significantly impaired throughout life both on verbal WM tasks and non- verbal WM (Numminen et al. 2001; Godfrey and Lee 2018), even if visuospatial working memory tends to be a relative strength compared with verbal working memory in those with DS (Schworer et al. 2022)

Carney and coll. (2013) used a cross-sectional developmental trajectories approach to assess verbal and spatial working memory (WM) in individuals with DS. A discrepancy between verbal WM (measured with the word list recall task) and spatial WM (measured with the Corsi Block task) was observed, with spatial WM performance aligning with mental age, while verbal WM performance was lower than expected for mental age. Both verbal and spatial WM followed a linear trajectory with chronological age, improving until around 20 years. These trajectories were similar to those of typically developing (TD) individuals during early adolescence but diverged in late adolescence and adulthood (Carretti et al. 2022).

1.1.5 Executive Functions

Executive functions (EF) are neurocognitive skills that serve as the foundation for early learning (Diamond 2013) and include working memory, shifting, planning and organisation, cognitive flexibility, monitoring and emotional control (Pennington and Ozonoff 1996; Miyake et al. 2000; Friedmanet al. 2006).

Subject with trisomy 21 showed poorer performance compared to TD subjects not only for the overall composites, but also separately for inhibition, shifting, and WM/STM. In particular, WM/STM and shifting are most severely affected, with large effects followed by inhibition with a medium effect (Tungate and Conners 2021).

Focusing on differences in EF throughout life preschoolers children Emotional Control can be classified as strength, Working Memory is a weakness (Loveall et al. 2017). During school-age the strongest skills appear to be Emotional Control and Organisation of Materials and the weakest concern Working Memory, Monitor, Plan/Organise and Shift (Daunhauer, Fidler, Hahn

et al. 2014; Lee et al. 2011, 2015; Loveall et al. 2017). The two age groups differed as regards Plan/Organise and Shift, domains in which the older children showed a more severe weakness. The greater difficulty in Shift and Plan/Organise in the older group may be because tasks in these domains get harder with age, or because the increasing demands at school make these difficulties more obvious to parents while they might previously have gone unnoticed (Loveall et al. 2017).

A study made by Van Deusen and coll. in 2022 highlighted the presence of two main profile involving EFs, one profile involved elevated scores in working memory only ("Working Memory Only" profile; 43% of sample) and a "Multi-Domain" profile that involved elevated scores in planning, inhibition, and working memory (57%). This last profile seems to be associated with the presence of congenital heart defects (Van Deusen et al. 2022).

Primary EFs (i.e., inhibition, shifting, and working memory) influences goal-directed behavior, or planning (Lanfranchi et al., 2010) and impairments in any specific primary domain of EF, or in higher-order EF (e.g., planning) have important implications for a variety of outcomes. For instance, inhibition is necessary for optimal functioning and participation in the school setting for children with DS (Daunhauer, Fidler, & Will, 2014). Difficulty with shifting can lead to missed learning opportunities or hyperfocus on irrelevant stimuli, which may translate to oppositional behavior around transitions (Will et al. 2016). Further, working memory is critical in the development and use of language and reading development (Abbeduto et al. 2007; Jarrold et al., 2009; Næss et al. 2011), academic achievement (Will et al. 2017), and employment (Tomaszewski et al. 2018) for individuals with DS.

Different studies showed how EFs is influenced by physical activity; in particular, EF showed an increase after 20 min of moderate physical activity (Vandoni et al. 2023). The main hypothesis, as suggested by Kasari and coll. in 1990, could be described by the arousal theory used for attention deficit hyperactivity disorder (ADHD) by which the symptoms of ADHD arise due to a reduction in executive control, caused by abnormalities in the structure, function and biochemical operation of the frontoparietal and frontostriatal neural networks (Johnson et al. 2009).

1.1.6 Adaptive Behaviours

The term 'adaptive behaviour' refers to skills that individuals use in their everyday lives and includes conceptual skills such as receptive and expressive language, reading, writing, math reasoning and understanding the concepts of time and money, social skills such as awareness

of others' thoughts and feelings, friendship skills, the ability to respect social rules, and social judgement and practical skills involve personal care, job responsibilities, money management and work task organisation (Schalock et al. 2010). Difficulties with these aspects define the grade of intellectual disability and this is why they are a crucial dimension to consider in individuals with DS (Schalock et al. 2010).

Infants and toddlers with DS (5–45 months old) showed a global impairment, compared with typically developing children, with difficulties across all domains already in the first year of life (Will et al. 2018). Studying cognitive profile, a varied picture emerged with strengths in Socialisation and weaknesses in Communication (Van Duijn et al. 2010). This profile remains fairly stable over time even if their standard scores declining as they grow older (Will et al. 2018; Spiridigliozzi et al. 2019) bringing to flatter profile (Van Duijn et al. 2010). Young children with DS that show a deceleration in adaptive trends involving both motor and communication skills (Will et al. 2018) and this profile seems to persist through adolescence and young adulthood before a decline in Communication that occurs beyond the age of 22 (Spiridigliozzi et al. 2019).

1.1.7 Developmental predictors and trajectories in children with DS

As described earlier difficulties in executive functions and adaptive behaviours may emerge as early as the first months of life. Therefore, it is important to be able to recognize early predictors and developmental trajectories so that early intervention can be made on them.

Studying developmental milestones, Locatelli and coll. in 2021 highlights that infants with DS acquired sitting at approximately 9 months of age, walking at approximately 24 months and sphincter control acquisition is approximately 44 months (Locatelli et al. 2021).

For the preschoolers (children from 3 to 6,99 years-old), sitting is a significant predictor of later gross motor development while in the School-age group (children from 7 to 16 years old), sitting becomes less important in predicting everyday life motor development. This could be explained because the earlier a child with DS started to sit, the earlier he/she started to walk, run, and have better general coordination or balance (Locatelli et al. 2021).

Motor milestones resulted to also be related to other developmental domains, indeed, in preschoolers, the age of onset of sitting showed a moderate correlation with later communication skills as described by parents (Locatelli et al. 2021). In our study a high correlation emerged between sitting and babbling and a possible explanation, as suggested by Iverson, could be that being seated permits deeper breathing and more consistent subglottal

pressure than a supine position (Iverson et al. 2010). Moreover, keeping the head upright alters the position of the spine and the vocal tract curve and the tongue falls to a more forward position in the oral cavity. This in turn enhances the production of Consonant–Vowel (CV) segments (Libertus et al. 2016).

In the School-age group, motor milestones showed a moderate relation with later cognitive and adaptive development both in children with DS (Locatelli et al. 2021) and in TD children (Hitzer et al. 2014, Van Batenburg-Eddes et al. 2013).

Considering language milestones, the mean age of acquisition of babbling is approximately 15 months, confirming that in children with DS the onset of babbling is delayed and continues into the second year of life. This milestone showed a moderate correlation to later everyday life communication in children between 3 and 6.99 years old, while in school-age participants it resulted moderately correlated with later communication and cognition (Locatelli et al. 2021).

Al least it is important to focus on the role of sphincter control in development. Preschoolers that have already acquired sphincter control have better motor, adaptive, and socio-emotional developmental levels while, in school age children, this milestone is a significant predictor of later motor, cognitive, communication, adaptive behaviour, and motor development (Locatelli et al. 2021). Sphincter control is a complex and highly distributed process that involves pathways at many levels of the brain, the spinal cord, and the peripheral nervous system and is mediated by multiple neurotransmitters (Fowler et al. 2008). The involvement of the prefrontal cortex on sphincter control might help us to understand its relationship with cognitive development. In fact the prefrontal cortex is fundamental for executive functions (i.e., working memory, planning, shifting, and inhibition), which are predictive of children's cognitive and adaptive functioning, e.g., (Tomaszewski et al. 2018). In this sense a more mature prefrontal cortex allows the child to have more mature regulatory and planning behaviours that have an impact both on sphincter control and on cognitive and interactive behaviours (Locatelli et al. 2021).

Switching from milestones to EF, it is well known that executive function are fundamental to planning, organising and monitoring everyday activities, and also for adaptive behaviour but their involvement changes at different ages.

In early childhood, WM, shifting and inhibition have been found to be related to communication (Mazuka et al. 2009; Kaushanskaya et al. 2017) and inhibitory control relates significantly to socialisation, helping an individual to avoid inadequate responses and to adjust to social norms (Diamond 2013; Benavides-Nieto et al. 2017).

The adaptive behaviour of adolescents aged 12-17 years needed to be more complex and articulated, and this probably meant a greater involvement of their EFs. In fact, Sabat et colleagues in 2020 highlight that WM predicted conceptual adaptive behaviour rated by parents, while inhibition and flexibility predicted conceptual adaptive behaviour rated by teachers. This difference may stem from the fact that different settings (home vs. school) make different demands on the child (Sabat et al. 2020). Porter et al. (2007) reported an association between the adaptive behaviours profile with frontal lobe abnormalities.

Another way to understand cognitive development is studying the developmental trajectories (Thomas et al. 2009), an approach based on the comparison of linear functions that link performance with age so that variability can be examined as a function of age.

Will and coll. (2018) explored developmental trajectories in adaptive functions (AF) over the first 3 years of life in DS, comparing to TD children, and they found that infants with DS up to 6 months old had similar communication skills to TD infants, while more pronounced deficits emerged in the former by 12 months of age. Difficulties in daily living skills and socialization emerged within the first year of life in the children with DS and remained consistent up to 45 months of age. In short, these age-related trends indicated that young children with DS had difficulty keeping pace with their TD peers in all areas of AF because their development tended to decelerate as they grew older, increasing discrepancies between DS and TD concerned motor and communication skills.

In the same group, Bunster and coll. (2022) identified three different trajectories, depending on the AF domain investigated. As Will and coll. they showed no differences in early life suggesting that the development of these skills might initially coincide with chronological age (CA). The DS group scored lower than the TD group on home living, self-direction, and social and motor skills, but their scores followed a similar linear trajectory, suggesting that these skills develop in line with mental age (MA), and that some factors (i.e., early support and intervention) might play a role. As for communication, community use, pre-academic, and health and safety skills, the two groups had similar scores at 15 months old, but the older children with DS scored lower than their TD peers, suggesting slower rates of development (Bunster et al. 2022).

1.2 Considering Down syndrome as a metabolic disease

In his studies, Lejeune hypothesized that DS could be considered a metabolic disease (Lejeune 1980) and this hypothesis was confirmed by different studies in literature.

In 2018 Caracausi and coll. conducted an analysis of the Nuclear Magnetic Resonance (NMR)-detectable part of the metabolome in plasma and urine samples, studying 67 subjects with DS and 29 normal subjects as controls selected among DS siblings, finding that there are significant alteration of several metabolites produced at the beginning or during the Krebs cycle (Caracausi et al. 2018). Further studies made by Antonaros and coll. in 2020 showed a clear discrimination of Down syndrome plasma metabolome that reaches up to 94% accuracy compared to plasma metabolome of general population (Antonaros et al. 2020).

This metabolic alteration involves different metabolic pathways and creates the wide spectrum of syndrome manifestations.

Studying hypoxemia, Donovan and coll. in 2024 found elevated heme metabolism and increased hypoxic signalling across the lifespan of subjects with Down syndrome, along with chronic overproduction of erythropoietin, elevated biomarkers of tissue-specific hypoxia, and hallmarks of stress erythropoiesis (Donovan et al. 2024) this condition may be influenced by the presence of obstructive apnea syndrome and may influence on intellectual developing and immune response to antigen.

Considering this metabolic dysregulation, in 2024 we studied zinc metabolism in children with T21 and it's role in their immunity status (Ramacieri et al. 2024). First we confirmed that zinc levels in Down syndrome are significantly low compared to general population as previously found in literature. Although this reduction seems not to be associated with immunity cells levels, it could influence the function of immunity cells as indicated by transcriptome map analyses made on lymphoblastoid cells, thymus and white blood cells. We found an increased expression of genes encoding for the metallothionein family and, even if not all gene expression ratios respect the 3:2 ratio suggesting that the alteration of these genes is due to a chain of regulatory events, this overexpression leads to a higher presence of metallothionein proteins that may cause a sequester inside cells of the circulating zinc (Ramacieri et al. 2024). Our analyses found also a lower expression of Ikaros zinc finger gene family in lymphoblastoid and thymus cell lines with a prevalence of 2:3 gene expression ratio. Ikaros proteins are important for lymphocyte development and other physiological processes. Focusing on SLC genes, whose role is to exporting zinc out or sequestrating cytoplasmic zinc into intracellular compartments when cellular zinc levels are elevated (SLC30 proteins) or importing the ion when cellular zinc is depleted (SLC39 proteins), 4 genes of the SLC30 family and 8 genes of the SLC39 family are DEGs. A higher prevalence of overexpressed SLC30 genes and underexpressed SLC39 genes is found. These data, found in the thymus transcriptome map, may suggest that there is an increased intracellular zinc level that could cause a decrease of extracellular zinc concentration (Ramacieri et al. 2024).

1.3 One-carbon pathway alterations in Down syndrome

One-carbon (1C) metabolism, is a universal metabolic process compartmentalized among the cytoplasm, nucleus, and mitochondria (Clare et al. 2019) that serves to activate and transfer 1C units for biosynthetic processes including folate metabolism, purine and thymidine synthesis, homocysteine remethylation and the trans-sulfuration pathway (Ducker and Rabinowitz 2017). In this pathway one-carbon groups at different oxidation states are transferred for biosynthesis of DNA through purine and thymidylate generation and for amino acid homeostasis, antioxidant generation, and epigenetic regulation (Lyon et al. 2020).

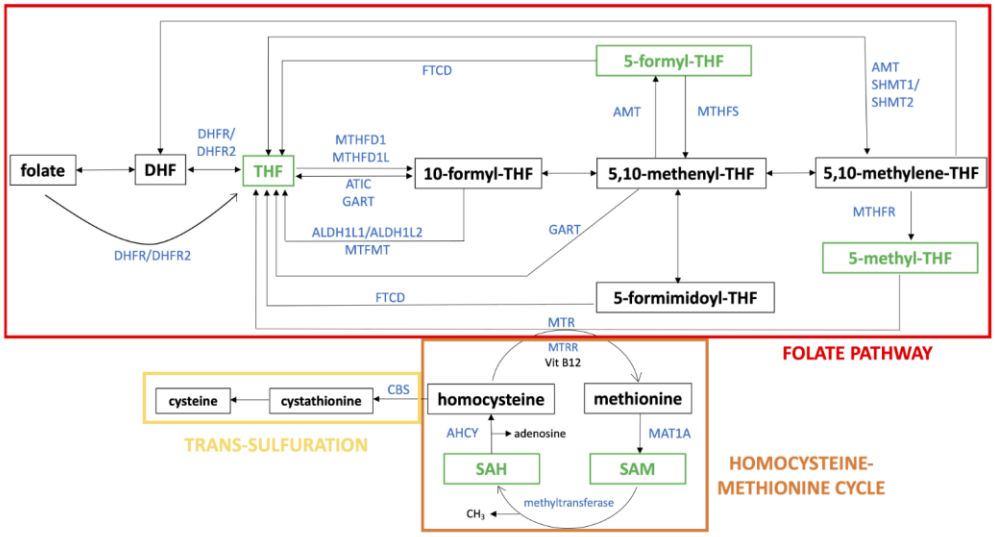

Figure 1 One-carbon pathway. *The figure shows a schematic representation of folate pathway (in red), homocysteine-methionine cycle (in orange) and trans-sulfulration pathway (in yellow), that are part of one-carbon pathway. The metabolites analyzed in our study are reported in green and the enzymes directly involved in their production or transformation are indicated with the official symbol reported in Gene NCBI (https://www.ncbi.nlm.nih.gov/gene). Alternative forms of enzyme are shown together separated by "/". The schematic representation was realized starting from "One carbon pool by folate - Homo sapiens" of KEGG pathway database (https://www.genome.jp/kegg/pathway.html) (Figure by Vione et al. 2022)*

In his 1979 pivotal study Lejeune, comparing DS with several metabolic diseases, hypothesized that a disturbance of the one-carbon cycle could occur in DS (Lejeune 1979). He thought that trisomic cells were intoxicated by an excess of gene products, which was caused by the presence of the additional Hsa21 (Lejeune 1990), he investigated the genes located on Hsa21 and he found that several enzymes involved in the one-carbon metabolism were encoded by genes located on this chromosome, in particular: cystathionine β-synthase (CBS) and

phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase (the three subunits of GART enzyme). Moreover, the gene for the main transporter of folate, solute carrier family 19 member 1 gene (*SLC19A1*), is also located on Hsa21 (Vione et al. 2022). The overexpression of these genes has been observed and causes a cascade of metabolic disturbances: low concentrations of folates, methionine, homocysteine, S-adenosyl methionine and serine and high levels of cysteine, cystathionine and methylated DNA (Pogribna et al. 2001, Lejeune et al. 1992, Gueant et al. 2005).

One-carbon metabolism was considered to be imbalanced in subjects with DS by several authors (Lejeune 1979, Rosenblatt et al. 1982, Peeters et al. 1995, Song et al. 2015, Funk et al. 2020). In particular, these subjects commonly present lower blood levels of vitamin B12 (or cobalamin) and folic acid with increasing age and lower erythrocyte folates and homocysteine (Hcy) compared to healthy controls (Ueland et al. 1990, Obeid et al. 2012, Song et al. 2015, Song et al. 2020).

The increased activity of CBS in children with DS significantly alters homocysteine metabolism such that the folate-dependent resynthesis of methionine is compromised. The decreased availability of homocysteine promotes the well-established "folate trap," creating a functional folate deficiency that may contribute to the metabolic pathology of this complex genetic disorder (Pogribna et al. 2001).

Vitale and coll. recently demonstrated the protective effect against MTX toxicity mediated by certain folate derivatives in human euploid and T21 fibroblast cell lines. Pogribna and coll. in 2001 showed that the addition of methionine, folinic acid, methyl-B(12), thymidine, or dimethylglycine to the cultured trisomy 21 lymphoblastoid cells improved the metabolic profile in vitro but Vitale and coll. showed that treatments with 5-methyl-tetrahydrofolate (5-m-THF, 5-methyl-THF) and 5-formil-tetrahydrofolate (5-f-THF, 5-formyl-THF, folinic acid) have significant protective effects on both euploid and T21 cells during MTX treatment (Vitale et al. 2019). Tetrahydrofolate (THF) is the metabolic active form of folates in cells and it serves as one-carbon carrier in most folate-mediated reactions (Zheng and Cantley 2019). It is the folate form most interconnected in the folate pathway and it is the product of ten enzymatic reactions as shown in Figure 1; 5-m-THF is the most abundant folate form in blood circulation and the only form able to cross the blood-brain barrier; it is formed by methylenetetrahydrofolate reductase (MTHFR) enzyme, starting from 5,10-methylene-THF (Caracausi et al. 2024). 5-f-THF is a reserve of one-carbon units in cells, and it does not play a direct biosynthetic role (Ducker and Rabinowitz 2017).

1.4 One-carbon pathway alterations and intellectual disability

There are numerous studies in the literature linking the one-carbon cycle with central nervous system development and its role may be explained through several mechanisms:

1) Methionine, folate, or B12 deprivation reduces cell proliferation due to blocks in cell cycling (Huang et al. 1999, Yang et al. 2016, Battaglia-Hsu et al. 2009, Shiraki et al. 2014)
2) Nutrient deficiencies can significantly impact early brain development often by hindering processes such as myelination, dendritic arborization, and synaptic connectivity that are crucial during early life stages (Lovblad et al. 1997). For instance, a deficiency of folates and vitamin B12 can impair myelin synthesis due to decreased methylmalonyl-CoA mutase (MUT) activity, leading to the incorporation of abnormal fatty acids into neuronal lipids (Briani et al. 2013). Myelination disruptions can severely affect central nervous system function by altering conduction speed across multiple systems (Black, 2008; Irvine et al. 2022) and this demyelination process can result in subacute combined degeneration (SCD) of the spinal cord and brain atrophy (Nyaradi et al. 2013), manifesting among other things as a loss of vibration and proprioception sensation.
3) Folates play a crucial role in influencing neuronal function, including vesicular transport, cell polarity, and plasticity, all of which can potentially affect cognition (Santos et al. 2020).
4) Neurotransmitter levels, such as serotonin, dopamine, norepinephrine, and acetylcholine, may be altered, leading to neuroanatomical, neurochemical, or neurometabolic changes (Black, 2008). Methionine serves as a precursor to S-adenosyl-L-methionine (SAM), a universal methyl donor that plays a critical role in metabolic pathways associated with the synthesis of hormones, neurotransmitters, nucleic acids, and proteins, especially in the brain (Irvine et al. 2022, Caracausi et al. 2024).

The association between low folate or B12 levels and an increased risk of neural tube defects (NTDs) is well-documented as well as their impact on both health and cognitive outcomes in children (Smithells et al. 1976 and 1980; Tolarova, 1982; Scholl et al. 1996; Czeizel, 1996; Shaw et al. 2009; Crider et al. 2011; Bestwick et al. 2014;). Proper neural tube closure depends on gene silencing through DNA methylation, and inadequate DNA methyltransferase (DNMT) activity, along with methylation cycle inhibitors, can impair this critical process (Greene et al. 2011). For these reasons the Centres for Disease Control and Prevention (CDC) in the United States recommends that women who previously experienced NTDs in pregnancy supplement 4 mg of FA per day (Centres for Disease Control 1990).

In the fetus, folate is essential for various critical processes, including cellular proliferation, neural stem cell proliferation and differentiation, reducing apoptosis, and altering and maintaining DNA synthesis (Darnton-Hill and Mkparu, 2015; Nyaradi et al. 2013; Anthony, 2007). The refinement of neural connections begins significantly around 24 weeks after conception and continues into the perinatal period (Raybaud et al. 2013; Levitt, 2003). As we have seen before, through the epigenetic mechanism of DNA (hypo)methylation, folate deficiency during pregnancy may modify gene expression, leading to long-lasting changes in the biological programming of brain development (Nyaradi et al. 2013). Indeed, folate insufficiency is associated with a reduction in the overall number of cells in the fetal brain (Craciunescu et al. 2004; Xiao et al. 2005) and decreased fetal brain weight (Middaugh et al. 1976). This was further demonstrated by Ars and coll. in 2019, who found that brain volumes were smaller in the low-folate group compared to the normal-folate group, while neither prenatal plasma B12 nor homocysteine levels predicted any brain volume outcomes (Ars et al. 2019).

Folate levels during pregnancy seems to affect child's neurodevelopment also after birth influencing the structure, cognitive scores and behavioural developing.

Folate insufficiency, particularly when plasma folate levels drop below 8 nmol/L, has been linked also to a global effect on postnatal brain development in offspring, characterized, as previously described, by an overall reduction in brain volume rather than a decrease in specific brain regions (Ars et al. 2019). Brain growth is particularly rapid during the first 2 years of life, especially in the cortex, with a peak period for synapse development and significant brain growth in humans occurs between 34 weeks post-conception and 2 years of age (Levitt, 2003; Huttenlocher and Dabholkar, 1997) and this is linked to higher-order thinking. Myelination of the brain, which is most concentrated from mid-gestation through the second year of life but continues through puberty, may also be vulnerable to vitamin B12 deficiency. In infants, such deficiency has been associated with demyelination and brain atrophy (Wighton et al. 1979; Lovblad et al. 1997).

Notably, prenatal folate levels have been shown to predict performance in specific cognitive areas, as measured by the NEPSY-II, such as memory/learning, visuo-spatial subdomains and language (Ars et al. 2019). Even if concerning the language part some studies found that higher maternal tHcy levels to be associated with better scores for verbal fluency (Veena et al. 2010). In humans, maternal folate deficiency has been associated with poorer performance on neurodevelopmental tasks during infancy (Del Rio Garcia et al. 2009) and childhood (Veena et al. 2010); children with prenatal homocysteine levels exceeding 9.1 μmol/L had significantly lower IQ scores (by 7 points) at age 6 years (Ars et al. 2019). This elevated prenatal

homocysteine level, coupled with lower prenatal plasma folate, was also associated with poorer performance in several cognitive domains at age 7 years (Ars et al. 2019).

Roza and coll., in 2010, found that children of mothers who did not use folic acid supplements during early pregnancy had a higher risk of emotional and behavioural problems at 18 months of age, as reported by the parents (Roza et al. 2010). These issues persisted, with emotional problems specifically observed at age 3 years (Steenweg-de Graaff et al. 2013). Furthermore, Schlotz and coll. (2010) reported that low maternal erythrocyte folate concentration (RCF) in early pregnancy was associated with behavioural problems, such as hyperactivity and peer problems, in 9-year-old children, as reported by mothers using the Strengths and Difficulties Questionnaire (SDQ).

If this deficit manifests itself in the first months of life, the symptoms described do not differ much from those previously described. In fact, infants born to mothers with sufficient vitamin B12 levels have adequate stores of the vitamin to sustain them for the first several months postpartum, with deficiency rarely occurring before about 4 months of age (Black, 2008). However, infants of vitamin B12-deficient breastfeeding mothers or those receiving low amounts of animal-source foods may become vulnerable to vitamin B12 deficiency between 6 and 12 months of age. Neurological symptoms associated with this deficiency primarily affect the central nervous system (Adhikari et al. 1985) and, in severe cases, can lead to brain atrophy, characterized by abnormal pigmentation, hypotonia, hepatosplenomegaly, sparse hair, food refusal, anorexia, failure to thrive, and diarrhea (Black, 2008). Several studies have described developmental retardation and “infant tremor syndrome” in 4- to 11-month-old infants of vegetarian mothers from India (Garewall et al. 1988; Srikantia et al. 1967). Additionally, four case studies from the United States reported symptoms such as lethargy, irritability, and developmental delay (Black, 2008). However, evidence from the treatment of children with vitamin B12 deficiency shows that even a short duration of vitamin B12 administration (e.g., a few days) can lead to significant improvements in functioning (Kopyta et al. 2006).

Progressing through life, vitamin B12 deficiency has been associated with various neuropsychiatric syndromes, including psychosis, mania, depression, and chronic fatigue syndrome (Briani et al. 2013) and there are also involved in cognitive impairment (Little 2018, Yukawa et al. 2002). The children raised on omnivorous diets from birth obtained better scores on most cognitive assessments than children raised on macrobiotic diets, regardless of their cobalamin status during adolescence. Many of the adolescents raised on a macrobiotic diet early in life had poor cobalamin status during adolescence, even if they were consuming an omnivorous diet later in life, illustrating the difficulty in restoring cobalamin status following early vitamin B12 deficiency (van Dusseldorp et al. 1999). After vitamin B12 therapy there was a rapid improvement of symptoms such as apathy and decreased activity. However, many

infants continued to experience developmental delays several months after initiation of treatment (Higginbottom et al. 1978, Wighton et al. 1979, Stollhoff et al. 1987).

In adults, deficiencies in cobalamin and/or folic acid have been linked with an inverse relationship to symptoms of depression (Tiemeier et al. 2002, Hickie et al. 2005; Sachdev et al. 2005; Gomez-Pinilla 2008) while elevated Hcy levels have also been linked to depressive symptoms. Furthermore, SAM has been used effectively as a treatment for depression (Mischoulon et al. 2002).

Moreover, folate and Vitamin B12 deficiencies are in both healthy elderly individuals and patients with Alzheimer's disease, low levels of B12 have been correlated with decreased cognitive function (Whyte et al. 2002). Elevated plasma homocysteine (Hcy) levels have been associated with lower intelligence test scores in the elderly (Bottiglieri et al. 2000; Duthie et al. 2002; Tolmunen et al. 2004) and a risk factor for dementia (12% to 31%) (Smith et al. 2018). A meta-analysis made by Whang and coll. in 2022 supports the hypothesis that early intervention with long-term higher intake of folate may provide benefit on cognitive function indeed slows cognitive decline in older adults (Whang et al. 2022) but there are no certain data in the literature on vitamin B12 or B6 intervention (Morris et al. 2005; Bailey et al. 2020).

1.5 One-carbon pathway alterations and intellectual disability in Down syndrome subjects

Few works in literature tried to associate alterations the level of metabolites belonging to one-carbon pathway to cognitive scores. In particular, Gueant and coll. in 2005 showed that in children with Down syndrome Hcy level above 7.5 mmol/l was associated with lower IQ scores (Gueant et al. 2005).

In 2021 Antonaros and coll. studied the influence of Hcy, vit B12, creatinine, folic acid and uric acid on cognitive scores in children with DS and they confirmed the trend ween by Gueant. In particular, in children between 7 and 16 years old, the non-verbal area has an increased moderate correlation with Hcy > 7.35 µmol/L (very similar to Gueants threshold) while the verbal area's correlation had a weak streght above the same threshold (7.35 µmol/L) suggesting that different environmental variables (ex. family environment, school education, speech therapy, external stimuli, etc.) can influence more the verbal area rather than the non verbal one (Antonaros et al. 2021).

The negative association of Hcy levels with cognitive scores confirmed literature data that reported this metabolite as an early marker of cognitive impairment in the elderly population and as increased in psychiatric phenotype (Antonaros et al. 2021).

Moreover, Antonaros and coll. highlight that, in children between 7 and 16 years old, a decrease of vitamin B12 levels under 442 pg/mL is correlated with a worse cognitive score and this threshold could be taken into account in the DS population which might have greater vitamin requirements.

Considering the possible role of metabolites belonging to this cycle, few trials have been conducted over the years to study the effects of possible folic acid (or similar) supplementation in infants with DS.

The first trial was proposed by Lejeune and colleagues in 1989 studying the medication with folinic acid of trisomic patients affected by severe infantile psychosis severe Alzheimer-like regression. They found a highly significant dose/effect correlation but this study did not meet current standards for clinical trials (Blehaut 2010).

In 2008 a randomized trial with antioxidant and folinic acid supplements showed no significant differences in developmental outcomes between those who received the supplements and those who did not. Likewise, there were no notable differences between the groups in the biochemical outcomes measured. Adjusting for potential confounders did not alter the results. This study offers no evidence to support the use of antioxidant or folinic acid supplements in children with Down syndrome (Ellis et al. 2008).

Blehaut and coll. investigated in 2010 the effect of oral folate supplementation (daily dose of 1.0+/-0.3 mg/kg) on cognitive functions in DS children, aged from 3 to 30 months, for 12 months. The intent-to-treat analysis (113 patients) did not show a positive effect of folic acid treatment. However, it identified important factors influencing treatment effect, such as age, sex, and concomitant treatments, including thyroid treatment in particular. Indeed, the positive effect of folic acid on developmental age was particularly strong in patients receiving concomitant thyroxin treatment (59.5% vs. 41.8%, $p<0.05$).

Last trial based on one-carbon metabolites supplementation was made by Mircher and coll. in 2020. They studied folinic acid and L-thyroxine supplementation in 143 DS infants aged 6-18 months assigning them to one of four treatments: placebo, folinic acid, L-thyroxine, or folinic acid +L-thyroxine, administered for 12 months. Even this trial does not support the hypotheses that thyroxine and/or folinic acid improve development of young children with DS or are synergistic.

2. Aims of this project

DS is a condition involving several clinical aspects. One of the aspects of greatest concern to families and clinicians is, without a doubt, the constant presence of a variable range of intellectual disability. The latter is present in all individuals with the condition and limits the life and autonomy of these patients.

Several studies have already involved different aspects associated with this syndrome (e.g., cardiovascular aspect) and have allowed these patients to have a much longer life expectancy than expected a few decades ago and studying the cognitive aspect is therefore crucial to improving the lives of these patients and their families.

As described in the literature, Down syndrome is a condition associated with altered metabolism. As hypothesized in the past by several clinicians such as Prof. Lejeune, an alteration in the one-carbon cycle may underlie the cognitive delay associated with this condition.

On this basis, the purposes of this project are:

1) to increase knowledge about the cognitive profile of children with trisomy 21, understanding strengths, weaknesses and developmental trajectories in order to identify aspects on which to act;
2) to develop a machine learning approach capable of analysing large datasets containing clinical, metabolic, laboratory, and genetic data in order to analyse such a complex aspect as cognition;
3) to further our knowledge of metabolites associated with the one-carbon cycle, in particular with metabolites that have not yet been studied (eg. THF), in patients with trisomy 21 and to study possible associations between them and cognitive aspects.

3. Patients, materials and methods

Ethical approval for this study was obtained from the Independent Ethics Committee of the Area Vasta Emilia Centro (CE-AVEC), Italy (number: 39/2013/U/Tess). We obtained an informed written consent from all participants to collect urine and blood samples, cognitive data and clinical data. Concerning minors, the consent was collected from his/her parents. All procedures were carried out in accordance with the Ethical Principles for Medical Research Involving Human Subjects of the Helsinki Declaration.

All participants were recruited during the annual follow-up of the individuals with DS at the Day Hospital of the Neonatology Unit, IRCCS Sant'Orsola-Malpighi Polyclinic, Bologna, Italy. Children and adolescents were assessed at the Department of Developmental Psychology at the University of Padova. The assessment sessions lasted approximately 90 min.

3.1 Cognitive profiles in children and adolescents with Down syndrome

All procedures, along with supplementary files and tables, are detailed described in Onnivello and colleagues work of 2022a.

3.1.1 Participants

The study concerned 72 children and adolescents with DS (males, n = 41). Their mean chronological age (CA) in months was 134.38 (SD = 31.24, min = 85.00 and max = 195.00). The inclusion criteria were a diagnosis of DS with homogeneous or mosaic trisomy 21 and a CA ranging between 7 and 17 years. This age range was chosen because it coincides with the time when individuals with DS reach their maximum developmental level, usually with no decline due to genetic or environmental factors. Trisomy 21 was confirmed by a karyotype analysis with amniocentesis during a prenatal screening, or by a blood sample after birth. None of the participants had mosaic trisomy. All participants were attending mainstream schools. Participants were recruited between November 2017 and February 2020.

3.1.2 Cognitive assessment

All participants were assessed with the Wechsler Preschool and Primary Scale of Intelligence-III, WPPSI-III17, a standardized method for measuring cognitive development for pre-

schoolers and young children (aged from 2.6 to 7.3 years). Although the WPPSI-III was designed for young children, it was used here to avoid any floor effect and because it was considered more in line with the supposed mental age of our sample.

The WPPSI has two versions, one for younger children (from 2.6 to 3.11 years old), and the other for older children (from 4 to 7.3 years of age). The former was used in the present study to ensure that all the children and adolescents could understand and complete the tasks. The latter would have led to a floor effect for several participants, which would have told us nothing about their skills. We also judged it more appropriate to derive performance indices from a test designed for younger children, rather than to obtain them statistically from a floor performance.

Although we chose to administer subtests intended for a younger age range, these tests could be used with children in the older age range to derive AE scores from the normative data. The version for younger children includes 5 subtests: Receptive Vocabulary, Picture Naming, Information, Block Design, and Object Assembly. In Receptive Vocabulary (which assesses receptive language), respondents are asked to look at a group of four pictures, and to point to the one the examiner names aloud. In Picture Naming (designed to measure expressive vocabulary), they have to name pictures shown one at a time in a stimulus booklet. In the Information subtest (for assessing a child's ability to acquire, retain, and retrieve general factual knowledge), respondents are asked questions testing their general knowledge. The Block Design subtest (which measures the ability to analyse and synthesize abstract visual stimuli, and to form non-verbal concepts) involves participants having to reproduce models with a set of blocks. Finally, there is Object Assembly (which assesses visual-perceptual regulation, and the ability to analyse and synthesize an abstract design), where participants are shown pieces of a puzzle in a standard arrangement and asked to fit the pieces together to form a given figure. The minimum score that a child could obtain in each subtest was 0, while the maximum was 38 for Receptive Vocabulary, 30 for Picture Naming, 34 for Information, 40 for Block Design, and 37 for Object Assembly. The scores obtained in the subtests were used to calculate a Verbal Index (which includes Receptive Vocabulary, Picture Naming and Information), and a Non-Verbal Index (comprising Block Design and Object Assembly). A Total Index can be calculated as well. Using a test standardized for younger children prevented us from considering standard scores, so AE scores were used instead.

The norms reported in the WPPSI- III manual were used to convert raw scores into AE scores. The normative data do not cover all the range of raw scores, however, so—for each subtest—we calculated a regression slope that enabled us to estimate the missing values with the following formula: AE score = slope × raw score + intercept. Since AE scores are influenced by CA, they were partialized for CA, and their residuals were used in the analyses. We opted to report AE scores in the descriptive statistics, however, as they are more readily interpretable.

Participants' developmental history. Caregivers provided family background and information on their children's development, including any medical conditions, when they reached the main milestones, and whether they attended any intervention programs. For the purposes of the present study, we considered the age when they reached specific milestones (sitting, babbling, walking, and first words), medical conditions (heart problems, a history of heart surgery and OSA), and parents' education level.

3.1.3 Analysis plan

All analyses were performed using R (Version 4.0.0).

To explore participants' overall cognitive profile, descriptive statistical analyses were conducted on the WPPSI verbal and non-verbal indices and subtests, then two ANOVAs were run on the scores obtained, one for the indices, and the other for the subtests.

To identify any subgroups with different cognitive profiles, a cluster analysis was run using the WPPSI Verbal and Non-Verbal Indices. Cluster analysis is an exploratory statistical method used to identify naturally-occurring groups or patterns of responses in a given set of measures or scales. Participants were empirically sorted into groups based on their relative similarities to one another on the measures considered (Henry et al. 2005). AE scores for the two indices were partialized for CA, and their residuals were used in the analyses. The residuals then underwent hierarchical cluster analysis, using squared Euclidean distances to distinguish the clusters. The agglomeration method was used because the Agglomerative Coefficient (AC) indicated that Ward's method was the one capable of identifying the strongest clustering structures ("Average" AC 0.89; "Single" AC 0.68; "Complete" AC 0.95; "Ward" AC 0.97; where values closer to 1 suggest a more balanced clustering structure, and those closer to 0 suggest less well-formed clusters). The "NbClust" package in R was used to validate the results of clustering analysis. Since this package provides an exhaustive list of validity indices for estimating the number of clusters in a data set (Charrad et al. 2015) , it was possible to compare the clusters resulting from the hierarchical cluster analysis with 30 fit indices. A majority rule

approach was considered to facilitate the choice of clusters in the real data sets (Charrad et al. 2015). To further confirm the results, the "tidyLPA" package in R was used to check the fit statistics on models with one to four clusters. The Bayesian Information Criterion (BIC) (Schwarz 1978) , the Entropy value, and the Bootstrapped Likelihood Ratio Test (BLRT) were considered. When the BIC is applied, lower values indicate a better fit. The Entropy value gives an indication of a model's classification quality, with values ranging from 0 to 1; higher values indicate a better classification quality (Celeux and Soromenho 1996), and values above 0.80 are generally assumed to indicate an adequate classification quality Jung and Wickrama 2008). The BLRT compares the improvements in fit between neighbouring class models (i.e., a model with k clusters to a model with k−1 clusters), generating a p value that is useful for establishing whether including one more class leads to a statistically significant improvement in the fit.

Two repeated-measures ANOVAs were used to explore the profiles between and within clusters, one considering the indices, the other considering the subtests, with Cluster as the between-subjects factor and Index/Subtest as within-subject factors.

When the assumption of sphericity was violated in the ANOVAs, the Greenhouse–Geisser adjustment was applied to p values (reported as p [gg]). Post-hoc t-tests were two-tailed and the p values were corrected for multiple comparisons using the Bonferroni method (i.e., the value of alpha divided by the number of comparisons).

Cohen's d was calculated to ascertain the magnitude of the difference between the clusters at each session. We also report Bayes factors (BF10) expressing the probability of the data, given H1 relative to H0 (i.e., values larger than 1 are in favor of H1, and those smaller than 1 are in favor of H0). The cut-offs for the BFs are: "anecdotal" (BF < 3), "moderate" (BF > 3), "strong" (BF > 10), "very strong" (BF > 30), or "extreme" (BF > 100) (Jeffreys, 1961). ANOVAs were run with AE scores partialized for CA, and their residuals were used. These analyses were also run on AE scores with CA as the control variable to see for any differences emerged. The results led to the same conclusion, so those with the residuals are reported for consistency with the cluster analysis where these scores were used.

Finally, to test the association with other variables, the chi-squared test was run for categorical variables, and correlations for continuous variables. Since the three medical conditions considered were dichotomous variables (present vs absent), and so was parents' education level (≤ high school vs > higher education), the chi-squared test was conducted in these cases, while correlations were run for age on reaching milestones.

3.2 Executive functions and adaptive behaviour in individuals with Down syndrome

All procedures, along with supplementary files and tables are detailed described in Onnivello and colleagues work of 2022b.

3.2.1 Participants

One hundred parents/caregivers of individuals with DS took part in the study, after giving their informed consent. The sample was divided into two groups based on the children's age and education level: preschoolers (aged between 3 and 6.11 years) and school-age children (between 7 and 16 years old). There were 40 children in the preschooler group and 60 in the school-age group. Participants' characteristics are shown in Table 1 (see Onnivello et al. 2022b). No differences emerged between the groups in terms of sex, race, or the mothers' or fathers' education.

3.2.2 Measures

The BRIEF-P (Gioia et al. 2003) is a standardised rating scale designed to measure EFs in children 2– 5.11 years old. It was completed by the parents or caregivers for the children with DS. The BRIEF-P presents a series of 63 statements regarding a child's behaviour. For each statement, parents are asked to rate how often (never = 1, sometimes = 2, or often = 3) each type of behaviour has been a problem in the previous 6 months. Higher scores indicate more severe problems. The BRIEF-P yields T-scores (M = 50, SD = 10), which are standardised scores based on the age and sex of the individual being described. There are five scales: Inhibit, Shift, Emotional Control, Working Memory and Plan/Organise, which together give rise to three index scales: Inhibitory Self-Control (ISCI = Inhibit + Emotional Control), Flexibility (FI = Shift + Emotional Control) and Emergent Metacognition (EMI = Working Memory + Plan/Organise). A composite score is obtained as well, called the Global Executive Composite score. The BRIEF-P parent form has a good internal consistency (0.80–0.95) and a good test–retest reliability (0.78–0.90; Gioia et al. 2003). Following a procedure already used in the field (e.g. Lee et al. 2011; Daunhauer, Fidler, Hahn et al. 2014; Pritchard et al. 2015), the BRIEF-P

was used in the present study for children from 3 to 6.11 years old (preschoolers), as the items in the BRIEF-P are more appropriate than those in the BRIEF for children attending preschool. Raw scores from each of the scales and indexes were used to generate age-referenced and sex-referenced normative T- scores. In this study, CA was used to generate age-referenced T-scores. However, considering that normative data are up to 5.11 years old, for children aged between 6 and 6.11 years, T-scores were calculated referring to the normative data for the oldest age range, that is, 4–5.11 years.

The BRIEF 2 (Gioia et al. 2000) is a standardised rating scale designed to measure EFs in individuals aged 6–18 years. The BRIEF 2 parent form consists of 86 items. The rating format and T-score norms are the same as for the BRIEF-P. The BRIEF 2 contains BRIEF-P: Inhibit, Self-Monitor, Shift, Emotional Control, Initiate, Working Memory, Plan/Organise, Task-Monitor and Organisation of Materials. The scales are combined to calculate three indexes: Behaviour Regulation (BRI = Inhibit + Self-Monitor), Emotion Regulation (ERI = Shift + Emotional Control) and Cognitive Regulation (CRI = Initiate + Working Memory + Plan/Organise + Organisation of Materials + Task-Monitor). Finally, a General Executive Composite score is calculated from all the scales. The BRIEF 2 parent form has a good internal consistency (0.80–0.98) and a good test–retest reliability (0.72–0.88).

Both versions of the BRIEF have already been used successfully with parents of individuals with DS (e.g. Edgin et al. 2010; Loveall et al. 2017).

Adaptive behaviours were assessed with Vineland Adaptive Behaviour Scales (VABS), Second Edition –Survey Interview Form. The VABS-II – Survey Interview Form (Sparrow et al. 2005) is a semi-structured interview for parents/caregivers of individuals aged from birth to 90 years. It investigates adaptive behaviour across four domains: Communication, Daily Living Skills, Socialisation and Motor Skills (for ages 0–6 years only). The Communication domain contains three subdomains assessing how well an individual understands language (Receptive), produces language (Expressive) and understands how to use letters and words, as well as how to read and write (Written). The Daily Living Skills domain contains three subdomains concerning an individual's skills in eating, dressing and hygiene (Personal), household tasks (Domestic), and time and money management, technology and job-related skills (Community). The Socialisation domain contains three subdomains covering an individual's relationships (Interpersonal Relationships), recreational skills (Play and Leisure), and how an individual demonstrates sensitivity and responsibility (Coping Skills). The Motor Skills domain includes two subdomains concerning fine and gross motor skills. Items are scored on a 0–2 scale indicating the frequency with which an individual uses a given skill autonomously: usually (2), sometimes (1) or never (0). Raw scores are converted into standard scores (M = 100; SD = 15), and a composite standard score, the Adaptive Behaviour Composite, is obtained from the

standard scores in the four domains. High internal consistencies have been reported across all VABS-II domains (rs = 0.70–0.95), and a high inter-rater reliability has been reported for the Survey Interview Form (rs = 0.68–0.95).

3.2.3 Analysis plan

Descriptive statistics, Student's t-test, repeated-measures ANOVAs and regression-based curve estimates were used in the analyses.

First, to answer the question of whether preschoolers and school-age children with DS have the same strengths and weaknesses in EFs, descriptive statistics were calculated on T-scores, and the percentages of clinically elevated scores were recorded.

Student's t-tests were used to see how the children and adolescents with DS compared with the normative group to identify similarities and differences with respect to typical development.

Repeated-measures ANOVAs were run to describe the profile of strengths and weaknesses in EFs separately for each group (since the BRIEF-P and BRIEF 2 scales partially differ), considering first the indices and then the scales.

In this analysis, the Greenhouse–Geisser adjustment was applied to the P values (reported as Pgg) when the assumption of sphericity was violated. Post-hoc t-tests were two-tailed, and the P values were corrected for the analysis of multiple comparisons using the Bonferroni method (i.e. the alpha value was divided by the number of comparisons). Cohen's d was calculated to establish the magnitude of the effects, where the rule of thumb for effect sizes was as follows: d (0.01) = very small, d (0.2) = small, d (0.5) = medium, d (0.8) = large, d (1.2) = very large and d (2.0) = huge (Sawilowsky 2009). Only the five scales that the two tools have in common (Inhibit, Shift, Emotional Control, Working Memory and Plan/Organise) were considered when comparing the EF profiles of the preschoolers and school-age children, using repeated-measures ANOVAs. As done for EF, for the question of whether preschoolers and school-age children with DS have the same strengths and weaknesses in adaptive behaviour, descriptive statistics were calculated on standardised scores, along with the percentages of clinically elevated standardised scores for preschoolers and school-age children.

To elucidate the strengths and weaknesses in adaptive behaviour in the two groups, and to compare the two profiles, a repeated-measures ANOVA was run with Scale as the within factor and Group as the between factor.

Finally, to explore whether the relationship between EFs and adaptive behaviour is the same in preschoolers and school-age children with DS, bivariate correlations were run separately for the two groups, to examine the relationship between the EF indexes (BRIEF-P and BRIEF 2) and the children's adaptive behaviour (Vineland-II). Regression models were used to explore the combined effect of the EFs considered on adaptive behaviour. For each model, age was entered first, followed by the indexes, and each Vineland-II scale was the outcome variable.

Following a procedure already used in the field (e.g. Esbensen et al. 2021), when an index was found associated with a Vineland-II scale, further analyses were run to detect which scale of the index showed the strongest association.

3.3 Cross-sectional developmental trajectories in the adaptive functioning of children and adolescents with Down syndrome

All procedures, along with supplementary files and tables, are detailed described in Onnivello and colleagues work of 2024.

3.3.1 Participants

This study involved a total of 115 Italian children and adolescents with DS (males, n = 70): 44 preschool-age children and 71 school-age children. Participants' characteristics are reported in Table 1. The inclusion criteria were a diagnosis of DS with homogeneous or mosaic trisomy 21, and a CA ranging between 3 and 16 years. None of the participants had mosaic trisomy. None of the children had significant hearing or visual impairments. All participants were attending mainstream schools in accordance with the Italian disability inclusion laws. The data of some of the qualified 115 participants were also used in other studies: 77 in Antonaros et al., (2020, 2021), 105 in Locatelli et al. (2021), and 72 in Onnivello et al. (2022b).

3.3.2 Measures

Adaptive functioning

Adaptive functioning was assessed using Vineland Adaptive Behaviour Scales, second edition (VABS-II), for details see paragraph 3.2.2

Cognitive development

Assessing cognitive development in individuals with ID poses a number of problems in the choice of the right test (for a critical discussion, see Pulina et al., 2019). In fact, the use of age-appropriate tests often leads to a "floor effect" due to the cognitive delay in this population. For this reason, a procedure often used in these circumstances (e.g., Locatelli et al., 2021; Onnivello et al., 2022b) involves the use of tests conceived for younger children. This has provided sensitive measures of cognitive functioning in children and adolescents with ID; however, it is important to also consider motivational aspects and the material should be appropriate for both the age and cognitive level of participants. Following all these considerations, and because in the present study a broad age range was under consideration, it was impossible to use a unique sensitive cognitive test for the whole age range. To avoid a floor effect and have a more reliable and feasible measure, two separate tests were used, the Griffiths-III for younger children and the WPPSI-III for older participants.

Griffiths-III. The Griffiths-III scales (Green et al., 2016) constitute a direct measure of overall development designed for children aged between 0 and 5.11 years. The Italian adaptation (Lanfranchi et al., 2019) was employed for children aged between 3 and 6.11 years. The A Scale—Foundations of Learning—was considered a measure of MA. This scale assesses cognitive development considering fundamental learning prerequisites during childhood, such as basic cognitive skills, thinking, memory, visuo-spatial skills, and reasoning. It involves both verbal and nonverbal items, although it is not possible to differentiate those two aspects in separate scores. In populations with ID, the use of standard scores leads to a floor effect. For this reason, following a procedure broadly used in the field, we converted raw scores into AE scores using the test's normative tables in order to have an estimation of MA. However, it is important to acknowledge that, if on one side, this kind of score has as a reference point the typical developmental trajectory, this does not necessarily mean that children with ID have exactly the same performance as TD children (Maloney & Larrivee, 2007).

Wechsler Preschool and Primary Scale of Intelligence (WPPSI-III). For details see paragraph 3.1.2

3.3.3 Analyses

Statistical analyses were carried out with R software, version 4.0.3 (R Core Team, 2023). AF was assessed as a function of CA. This was done considering the developmental trajectory approach (see, for example, Carretti et al., 2022). This approach recommends that when analysing cross sectional data of a broad age range group to describe a developmental trajectory, it can be helpful to analyze functions that link the performance with age. Therefore, we chose to describe AF performance as a function of age in DS. Following a procedure previously used, we considered not only linear functions but also null and segmented regression models (Carretti et al., 2022). The last of these (also called piecewise regression models) enable multiple linear models to be fitted to data for different ranges of a dependent variable. The models were fitted using the "segmented" library (Muggeo, 2008) in R. The best-fitting model was chosen according to the Akaike information criterion (AIC, where lower is better; Akaike, 1973) and the significance of the p-value ($p < 0.05$).

Trajectories were explored with the following variables:

• standard scores for AF (AF-ST) as the dependent variable, and CA as the predictor; and

• AE scores for AF (AF-AE) as the dependent variable, and CA as the predictor.

In our analysis we considered both standard and AE scores because we expected different trajectories depending on the type of score considered. Moreover, in order to compare the impact on AF of CA and MA, the same approach was used considering AF-AE scores as the dependent variable, and MA as the predictor. In this case, only AF-AE scores were considered, since this was the only measure of AF comparable with cognitive MA.

Stepwise regression models were then run to explore which variable, CA or MA, better explained progression in AF. The dependent variable was the AF-AE score, and the independent variables were CA and MA.

Finally, in order to visually explore and compare AF-AE and MA trajectories against CA, we explored the MA trajectory as a function of CA. The null, linear, and segmented models were tested to see which one better described our data.

3.4 Machine learning based analysis for intellectual disability in Down syndrome

All procedures, along with supplementary files and tables, are detailed described in Baldo and colleagues work of 2023.

3.4.1 Dataset and preprocessing

The dataset used in this study was obtained during routine follow up visits from February 2014 to July 2019. Inclusion criteria were diagnosis of DS with homogeneous or mosaic trisomy 21 and age >2 years (yrs).

The DS clinical dataset here reported in Supplementary Table 1 (see Baldo et al. 2023) is composed of a collection of anonymized personal records regarding genetic, diagnostic, clinical, and auxological information. For details regarding the recorded data, we refer to previous works where subjects are indicated with the same DS subject code (Locatelli 2021 and 2022, Antonaros 2020 and 2021).

To perform a correct analysis of the data sample we preprocessed the data. This phase was composed of the following steps: (i) starting from Supplementary Table 1, we removed 72 variables, which were deemed unimportant or not informative due to missing values; (ii) we codified categorical attributes introducing a numerical encoding of the values signifying different classes (e.g., YES/NO becomes 0/1); (iii) attributes with signs or string not digestible by an ML algorithm (for instance, numerical attribute containing “>” symbols) were processed and cast to data type conform to the input values required by the model. After preprocessing steps, the dataset has 5 sets of variables on 106 subjects. Table 1 lists all final 109 variables in the dataset, and the associated datatype for each of the attributes. In particular, the acquisition of the development skill milestones was recorded with the month at which the subject started babbling (“Babbling”), sitting without support (“Sitting”), walking without assistance (“Walking”) and controlling sphincter and urination (“Sphincter control”), as reported by parents.

A general overview of our ML analysis workflow is shown in Figure 2.

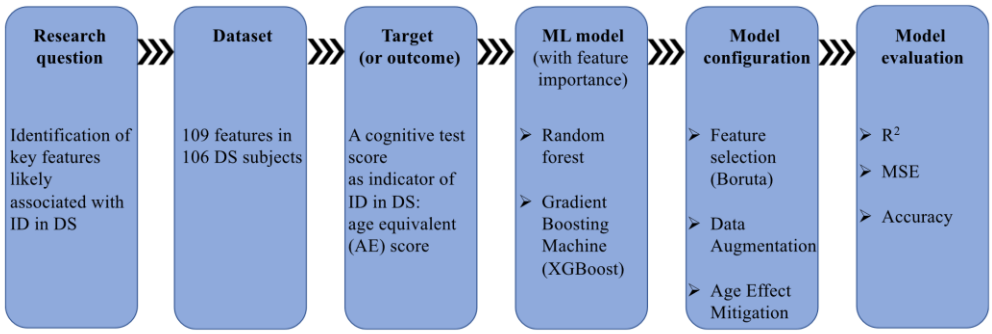

Figure 2. Overview of machine learning analysis workflow. *We have applied two tree-based machine learning (ML) models (Random Forest and XGBoost) to the research question: how to identify key features likely associated with intellectual disability (ID) in Down syndrome (DS). We analysed 109 features (or variables) in 106 DS subjects. The target (or outcome) of the analysis was the age equivalent (AE) score as indicator of intellectual functioning, impaired in ID. We applied several methods to configure the models: feature selection thought Boruta framework to minimize random correlation; data augmentation to overcome the issue of a small dataset; age effect mitigation to take into account the chronological age of the subjects. Figure by Baldo et al. 2023*

In our analyses the predictive target (or outcome) is a cognitive test score as indicator of ID in DS. Following a procedure broadly used in the field (Antonaros et al. 2020 and 2021, Pulina et al. 2019) in order to assess cognitive functioning in individuals with DS, tests more appropriate for the expected mental age instead of chronological age were used. This allowed us to avoid the floor effect (and the consequent lack of information) that is often present when tests appropriate for chronological age are used. In particular, the cognitive score was calculated starting from raw scores obtained from the Griffiths-III test (Green 2016) for DS subjects between 3 years and 6 years and 11 months and the WPPSI-III test (Wechsler 2002) for 7-year-old or older DS subjects. Raw scores were transformed into AE scores according to normative data. In order to consider comparable test scales between the two groups, for the scope of this analysis we considered only scale A ("foundations of learning") Griffiths-III test (column "A AE G" in Supplementary Table 1) and "total" WPPSI-III test AE scores (column "Tot AE W" in Supplementary Table 1). However, to take into account the subject's chronological age in the prediction of the outcome, the age effect mitigation was applied as explained below.

3.4.2 Machine learning models and model building

In the present study, we have applied two decision tree-based ML models (Random Forest and Gradient Boosting Machine) to identify key features likely associated with ID in DS.

Random Forest (RF) is based on training of multiple trees on random subsets of variables. The final prediction is the result of a voting system, where each tree returns a predictive outcome for an input instance and the majority output will be the overall returning value (Belgiu et al. 2016).

Gradient Boosting Machine (GBM) is another tree-based method. If RF builds decision trees in parallel, approximating different views (or subsamples) of the dataset, GBM builds models sequentially, gradually minimizing the loss function and consequently the overall training error. The final output is computed incrementally, i.e., the prediction of each decision tree is summed to obtain the overall outcome. Among the most popular GBM models available we choose XGBoost, which in recent years has been widely used to solve a variety of problems with a high level of accuracy (Ogunleye et al. 2020).

3.4.3 Feature importance

Feature importance was used to quantify how important each input attribute is for reaching a prediction. In our case it was used in both RF and XGBoost methods to identify which attribute is more correlated with the ID score, so as to guide future investigations.

Whenever we create a split while we train a tree model (both RF and GBM), this will be associated to an increase of the performance indicator. By keeping track of the cumulative increase, it is possible to estimate their impact on the tree behaviour indicating feature importance scores.

Feature importance scores are the key instrument for our analysis. For this reason, it is important to understand that, since most ML models (including the ones we use) operate on the basis of correlations in the data, a high feature importance is not an indicator of cause-effect relation. In the experimental results, we will display the feature importance as a bar plot, showing the ranking of the first ten variables with higher relevance based on their score.

It is also important to observe that the reliability of feature importance score is limited by the performance of the ML model itself. In other words, the importance score extracted from an inaccurate model may be misleading.

3.4.4 Data manipulation

In this section, we will present three methods for tackling different issues relative to the dataset used in our analysis (Fig. 2). Each of these methods refers to a specific problem that arose during the analysis.

Feature selection

Feature selection allows the model to focus on the most important features or on features whose importance is of statistical significance. By doing so, we reduce the risk of overfitting, and we obtain more reliable importance scores as a side effect.

We applied Boruta, a wrapper-based feature selection method to filter out a set of features that are relevant to the target variable (Kursa et al. 2010). It is implemented through the following steps: 1) The features are randomly shuffled and then stitch together with the actual feature matrix to form a new feature matrix. 2) The importance of the shuffled and actual features is obtained by inputting the newfeature matrix into the random forest. 3) The actual features with importance greater than the maximum importance of the shuffled features are retained. By iterating the above steps several times, the important features are identified by Boruta.

Data augmentation

To increase the ML models accuracy starting from a small sample size, we used data augmentation techniques. The number of data augments quadratically, going from n sample to ∑in-i by comparing pairs of input instances, where each new data entry is computed as the ratio between the variables pertaining to two subjects. With this step we are changing the question our model is designed to answer: rather than asking “given information about subjects what is the estimated AE score?”, we now ask “given information about ratios of input attributes for a pair of subjects, what is the ratio of their AE score?”. In scientific terms, the new question is weaker than the previous one, in fact given an oracle for the former it is possible to answer the latter while the opposite is not possible.

That said, the new question is still highly informative from the point of view of our main goal: if the ratio between (say) vitamin B12 for two subjects is predictive of the ratio between their AE scores, there is intuitively a chance the feature is involved in ID.

The question can be further simplified by focusing on the detection of the higher AE score in the pair of subjects. In this case, we ask "given information about ratios of input attributes for a pair of subjects, which subject has the higher AE score?". This method allows the formulation of the problem as a classification task, where the target is formulated as a category. More precisely, if we consider two subjects qi and qj, and the category associated with their comparison, ck: • ck = 1 if qi > qj; • ck = 2 if qi ≤ qj.

Age effect mitigation

In our analysis the outcome is a cognitive test score as indicator of ID in DS. In particular, we used the AE score of children with DS.

It is important to note that the chronological age of the subject is highly related to the target of the model and might distort the real importance of other features. Moreover, this argument can be made for each variable that is correlated to the chronological age of the children. In this situation, features strongly correlated with chronological age would naturally have a higher importance score in our model.

To address this issue, we have applied an age effect mitigation: in this way we avoid to overfit about the chronological age using age information to compute normalization factors (estimated using ML models, in particular artificial neural networks). The chronological age of the subject was used as a predictor of any other variable in our sample. In this case, we might face two scenarios: (i) the age of the subject is a good predictor of the variable. Therefore, all its values will be normalized to approximately one, meaning it will not be as informative during the training process; (ii) the age of the subject is not a good predictor of the variable, then the values of the variables will be re-scaled according to the predicted value.

3.4.5 Model evaluation

In order to have a reliable interpretation of the results, we needed to evaluate the ML models used to approximate the samples, since scarce performances would not allow for a trustworthy

analysis. To this end, we relied on commonly used performance metrics, such as: R2, to measure the goodness of the fit, and Mean Squared Error along with the accuracy score, to measure the error associated to the models.

To avoid incurring in overfitting problems, we relied of a K-fold cross validation approach (K = 10); thus, all the results provided regarding the metrics are specified as mean and standard deviation among the different validation splits.

3.5 One-carbon pathway metabolites are altered in the plasma of subjects with Down syndrome: Relation to chromosomal dosage

All procedures, along with supplementary files and tables, are detailed described in Vione and colleagues work of 2022.

3.5.1 Participants

A total of 339 subjects were selected for this study, including 285 subjects with DS and 54 subjects as control, selected among siblings of subjects with DS and without evidence of abnormal karyotype.

For this work, we considered in the DS group subjects with diagnosis of DS with homogeneous or mosaic T21, availability of an adequate amount of plasma to perform at least one ELISA assay and a similar mean age as close as possible to control group. Concerning control group, we considered subjects with an adequate amount of plasma to perform at least one ELISA assay and a similar mean age as close as possible to DS group.

3.5.2 Blood drawn and sample preparation

The blood samples selected were treated within two hours of collection and plasma samples contaminated by residual erythrocytes after the treatment were excluded.

For every collected sample, parents filled out a form with information about the current fasting state, last meal, consumed medications. Blood sample preparation

For DS group, two blood sample aliquots were collected in EDTA-coated blood collection tubes in the Neonatology unit. The first aliquot was sent to “Laboratorio unico Metropolitano” (LUM) of Maggiore Hospital (Bologna, Italy) for routine blood analyses of the DS group including folic acid and vitamin B12. The concentration levels in serum were obtained by chemiluminescent immunoassay by Beckman Coulter Immunoassay Systems (Beckman Coulter, respectively REF A98032 and REF 33000). The second aliquot was kept at room temperature and treated within two hours from blood draw for plasma isolation. For control group, one blood sample aliquot were collected in EDTA-coated blood collection tubes in the Neonatology unit. The second aliquot was kept at room temperature and treated within two hours from blood draw for plasma isolation. Plasma isolation for DS and control aliquots was performed as following. Sample was transferred to a new tube and centrifuged at 1,250 g for 10 min to separate corpuscular fraction from plasma. The plasma was isolated and centrifuged for a second time at 800 g for 30 min to eliminate residual debris. The supernatant was collected and transferred into new tubes avoiding contact with pellets or the bottom of the tube, divided in aliquots and rapidly stored in a −80◦C freezer. Every delayed treatment of the sample following its transfer to the university laboratory (> 2 h) was recorded, as one of the exclusion criteria from the analysis was sample treatment after two hours from the draw. The other one was evident contamination of plasma by hemoglobin due to hemolysis after the treatment. All procedures were conducted carefully to avoid contamination during the different steps and any anomalies like different plasma color or precipitates after centrifugation were noted and considered in further analysis.

One or more plasma aliquots were used for ELISA assays. For DS group only, one plasma aliquot was sent to LUM for the detection of Hcy plasma level by an automated chemistry analyzer AU 400 Beckman Coulter (Beckman Coulter, REF FHRWAU/FHRWR100/200/1000).

Concerning concentration values of folic acid, vitaminB12 and Hcy of DS group, 25% of data that are present in Supplementary Data Sheet 1 of Vione et al. (2022) can be found our previously published work (Antonaros et al. 2021).

3.5.3 Enzyme-linked immunosorbent assay

We evaluated the quantitative measurement of THF, 5- methyl-THF, 5-formyl-THF and SAH plasma concentrations using specific ELISA kits manufactured by MyBioSource (San Diego, California, USA) and SAM plasma concentration using a specific ELISA kit manufactured by Biovision (Milpitas,California, USA).“General Tetrahydrofolic Acid (THFA) ELISA assay,”“Human 5-methyltetrahydrofolate ELISA assay,” and “Enzyme-linked Immunosorbent assay for 5-formyl-THF (folinic acid)” MyBioSource kits are based on a competitive inhibition enzyme technique.

Regarding THF, specifications were: detection range, 1.23- 100 ng/mL; sensitivity, 0.57 ng/mL; intra-assay precision, Coefficient of Variation (CV) < 10%; inter-assay precision,CV < 12%; no significant cross-reactivity or interference between THFA and analogs was observed.

Regarding 5-methyl-THF, assay specifications were: detection range, 0.156-10 ng/mL; sensitivity, 0.094 ng/mL; intra-assay precision, CV < 8%; inter-assay precision, CV < 10%; no significant cross-reactivity or interference between 5-methyl-THF and analogs was observed.

Regarding 5-formyl-THF, assay specifications were: detection range, 117.2-30,000 pg/mL; sensitivity, <41.5 pg/mL; intra-assay precision, CV < 10%; inter-assay precision, CV < 12%; no significant cross-reactivity or interference between Folinic Acid (FA) and analogs was observed.

MyBioSource “Human S-Adenosyl-Homocysteine (SAH) ELISA assay” and Biovision “S-Adenosylmethionine (SAM) ELISA” kits employ a double antibody sandwich technique.

Regarding SAH, assay specifications were: detection range, 20 ng/mL-0.312 ng/mL; sensitivity, up to 0.06 ng/mL; intra-assay precision, CV ≤ 8%; inter-assay precision, CV ≤ 12%; no cross-reaction with other factors.

Regarding SAM, assay specifications were: detection range,0.39-25 ng/mL; sensitivity, < 0.234 ng/mL; intra-assay precision, CV < 8%; inter-assay precision, CV < 10%; nosignificant cross-reactivity or interference between SAM and analogs was observed.

Ninety-six well plates were used for all the assays and all standard and plasma samples were tested in duplicate. Due to preliminary studies, plasma samples used for the measurement of 5-methyl-THF were diluted 1:10 using Sample Dilution Buffer supplied by the kit and plasma samples used for the measurement of SAH were diluted 1:5 in Plates 1, 2, 3, and 4, and 1:3 in Plates 4 and 5 to avoid excessive dilution of the metabolite, using Sample Diluent supplied by the kit. The standard samples were provided by the kits and were reconstituted and serially diluted as suggested.

In order to perform the assays, the manufacturer instructions of each kit were followed, and the final spectrophotometric reading was carried out by microplate reader (Perkin Elmer Wallac 1,420 Victor 2 Multi-Label) set at a wavelength of 450 nm.

In order to create the standard curve for each ELISA assay, standard O.D. mean values were plotted on the x-axis and the known standard concentration values were plotted on the y-axis using Microsoft Excel and following manufacturer instructions. The transformation of standard concentration values in their logarithm (Log10) is required to build the standard curves in THF and 5-formyl-THF ELISA assays. The subtraction of the background O.D. mean value ("standard 8") from the O.D. mean values of the other standards and plasma samples is required in SAH ELISA assay. The transformation of standard O.D. mean values in their inverse (1/O.D. mean) is required to build the standard curves in the case of the SAM assay. The polynomial trend lines of each plot were created using Excel, and the resulting polynomial equations ($y = a + bx + cx1$) were used to determine metabolite concentrations of plasma samples using interpolation.

3.5.4 Statistical analyses

Statistical analyses were carried out with SPSS Statistics software (IBM, Version 25 for Mac OS X) and were performed using the data available in Supplementary Data Sheet 1. For all results, a $p < 0.05$ was considered statistically significant. An $r < 0.4$ was considered as weakly correlated, $0.4 < r < 0.7$ as moderately correlated and $r > 0.7$ as strongly correlated.

We used SPSS Statistics to perform a first descriptive analysis in order to obtain a general view of the data included in Supplementary Data Sheet 1 of Vione et al. 2022. and to highlight the presence of strong outliers in concentration level distribution. SPSS considers data as an outlier if it is outside the following ranges: above the 3rd quartile + 1.5 interquartile range or below the 1st quartile −1.5 interquartile range and indicates it with an asterisk in the graph.

To check if our data followed a normal distribution we used Kolmogorov-Smirnov test using Social Science Statistic software online (https://www.socscistatistics.com/tests/kolmogorov/default.aspx)

For each metabolite (THF, 5-methyl-THF, SAH, and SAM) we performed an unpaired student t-test between DS and control group using the "Graph Pad" t-test calculator online (https://www.graphpad.com/quickcalcs/ttest1.cfm), while for 5-formyl-THF levels, that resulted to be distributed in a way significantly different from a normal distribution, we performed Mann-Whitney test online (https://www.socscistatistics.com/tests/mannwhitney/).

The graphic reports of each subject's metabolite plasma levels in the study were created with GraphPad Prism software v.6.0 (San Diego, CA) (Figure 2 and Supplementary Figure 1).

In SPSS Statistics we used linear correlation to search for correlation between age and molecule levels and unpaired t-test to verify whether sex and fasting/non-fasting state might affect the main results.

SPSS Statistics was used to perform a linear correlation between the level of each molecule and the levels of all the other molecules. Partial correlation analyses checked for the effect of chronological age were used to investigate associations between the level of the involved molecules and other molecules.

The Heat Map figures (Figure 16 and Supplementary Figure 2 of Vione et al. work (2022)) representing metabolite correlations (THF, 5-methyl-THF, 5-formyl-THF, SAH and SAM) were generated using JMP Pro software (Version 14 of the SAS System for Mac OS X, SAS Institute Inc., Cary, NC, USA).

3.6 Analysis of metabolic and genetic imbalance of the homocysteine-methionine cycle in trisomy 21

All supplementary files are available upon request (paper submitted to the journal).

3.6.1 Case selection

A total of 106 subjects were selected for this study, including 58 subjects with DS and 48 subjects as control group (N), selected among siblings of subjects with DS and without evidence of abnormal karyotype.

For this work, we considered in the DS group subjects with a diagnosis of DS with homogeneous or mosaic T21, the availability of an adequate amount of urine to determine SAM and SAH metabolites, and we recorded whether the samples were collected in a fasting or not-fasting state. The urine samples were collected from subjects with a similar mean age as close as possible to the N group. Concerning the N group, we considered subjects who do not have pathologies, availability of an adequate amount of urine to determine SAM and SAH

metabolites, and similar mean age as close as possible to the DS group. The urine samples selected were treated within two hours of collection and with an apparent good physical state (clear urine after centrifugation).

DS group consists of 35 males (M) and 23 females (F) with a mean age of 12.72 years old (yrs) (standard deviation, SD=5.92) and age range from 3 to 28 yrs; the N group consists of 32 M and 16 F with a mean age of 14.42 yrs (standard deviation, SD=7.10) and an age range from 2 to 31 yrs. Concerning the N group, 20 subjects were siblings of 17 subjects in the DS group. For every collected sample, parents filled out a form with information about the current fasting state, last meal, concomitant diseases and consumed medications (Supplementary dataset 1).

3.6.2 Urine sample preparation

Preanalytical treatment of urine samples followed standard operating procedures (Bernini et al. 2011). All procedures were conducted carefully and in sterility to avoid contaminations.

Urine samples were collected in a sterile plastic cup with a lid and kept refrigerated at +4°C if immediate processing was not possible. They were treated within two hours of collection. The sample was transferred to a new tube and centrifuged at 2500 g for 5 min at +4°C. After centrifugation, filtration by a 0.20 µm cut-off filter was performed to avoid contamination of the metabolome with soluble molecules derived from cellular components. The filtered urine was transferred to sterile cryovials making 1.0 mL aliquots. All urine samples were rapidly stored in liquid nitrogen and ready for subsequent analysis.

3.6.3 Liquid Chromatography-Mass Spectrometry (LC-MS)/MS analysis

We employed an Agilent 1260 Infinity II LC system (Agilent technologies, Waldbronn, Germany) for chromatographic separation, consisting of a Multisampler (G7167A), quaternary 1260 Infinity II Flexible Pump (G7104C), and Multicolumn Thermostat (G7116A). The separation was carried out using a Zorbax Eclipse Plus C18 RRHD column (50 mm × 2.1 mm, 1.8 µm particle size, Agilent Technologies, Waldbronn, Germany). For mass spectrometric detection, the UHPLC column was connected with PEEK® tubing (0.005” ID × ca. 60 cm long) to a triple quadrupole Ultivo® LC/TQ system (G6465B). Chromatographic separation was performed isocratically with a mobile phase containing 0.1% v/v heptafluorobutyric acid and 10% acetonitrile (Sigma-Aldrich, Steinheim, Germany). The column was temperature-

controlled at 30 °C, the mobile phase flow rate was set at 0.4 mL min-1, and the injection volume was 1.0 µL. Instrumental parameters for the electrospray ionization tandem mass spectrometric detector (ESIMS/MS) were as follows: Nebulizer gas temperature: 350 °C; Nebulizer gas flow:10 L min-1; Nebulizer pressure: 35 psi; Sheath gas: 400 °C; Sheath gas: 12 min-1; Fragmentor voltage: 50 V; Collision energy: 20 eV; Capillary voltage: 3000 V. SAM and SAH were quantified using the mass transitions 399 → 250, and 385 → 136, respectively.

Urine was thawed, mixed by vortexing, and centrifuged for 15 min at 10000 ×g. To minimize matrix effects, 100 µL of the urine supernatant was diluted with 900 µL water. The combination of this dilution with the employed small injection volume (1.0 µL) was sufficient to eliminate the matrix effects as indicated by recovery experiments where recoveries were found within the range of 85-115%. The limit of detection was 0.01 µM (calculated based on the S/N = 3 method).

3.6.4 Comparison between plasma and urine levels of SAM and SAH

A comparison between the SAM and SAH levels in urine (uSAM and uSAH) to a different biological fluid as plasma was performed. In particular, SAM and SAH dosages in plasma (pSAM and pSAH) of DS and N subjects obtained by ELISA assays and recently published (36) were used for the subjects of which we have urine quantifications (Supplementary dataset 1). pSAH levels were available for 30 out of the 58 DS subjects and for 15 out of 48 N subjects, while pSAM levels were available for 8 out of 58 DS subjects and for 11 out of 48 N subjects.

Plasma values were converted from ng/mL to nM (Supplementary dataset 1) according to the formula [(ng/mL)*1000]/MW, where MW is the molecular weight (g/mol). The MW of SAM is 398.44 g/mol, MW of SAH is 384.412 g/mol.

3.6.5 Statistical analyses

Statistical analyses were carried out with SPSS Statistics software (IBM, Version 28 for Mac OS X) and were performed using the data available in Supplementary dataset 1. For all results, a $p<0.05$ was considered statistically significant. An $r< 0.4$ was considered as weakly correlated, $0.4<r<0.7$ as moderately correlated and $r>0.7$ as strongly correlated.

In order to choose the correct test to perform the statistical analysis, we first checked whether our data followed a normal distribution using the Kolmogorov-Smirnov test of the online Social Science Statistics software (https://www.socscistatistics.com/tests/kolmogorov/), then we analyzed the influence of age, sex, and fasting status on metabolite levels using SPSS statistical software; specifically, we performed the linear correlation between age and metabolite levels to see if there was a link between them, the unpaired t-test to see if sex affects urinary and plasma metabolite levels, and the Kruskal-Wallis test to see if fasting/non-fasting status could influence the main results.

Once the previous analyses were performed, we investigated whether there were different distributions of SAH and SAM levels in the different groups. First, we performed in both DS and N groups an unpaired t-test using SPSS software to detect any differences in the distribution of SAM and SAH levels in plasma and urine samples; later, we performed the same test to highlight any differences in metabolite levels (SAM and SAH in urine samples and SAM and SAH in plasma samples) between the DS and N groups.

Finally, we performed a partial correlation checked for the effect of chronological age between SAM and SAH levels in the urine samples of the DS and N groups to test whether the correlation found by Vione and coll. 2022 is also present in the urine samples.

3.7 One-carbon pathway metabolites involvement in cognitive development in children with DS

All supplementary files are available upon request

3.7.1 Case selection

A total of 83 subjects with DS were selected for this study of which 30 were children between 3-6.99 years old and 53 were children between 7 and 16.99 years old. Inclusion criteria were diagnosis of DS with homogeneous or mosaic T21, availability of an adequate amount of plasma to perform at least one ELISA assay and age between 3 and 16 years old.

3.7.2 Blood Sample Preparation and Enzyme-linked immunosorbent assay

Blood sample preparation procedures and ELISA assays where the same described in paragraph 3.5.2 and 3.5.3

3.7.3 Cognitive evaluation

Cognitive data were assessed using the same tests and procedures described in paragraphs 3.1.2, 3.2.2 and 3.3.2.

3.7.4 Statistical analyses

Statistical analyses were carried out with SPSS Statistics software (IBM, Version 25 for Mac OS X) and were performed using the data available in Supplementary Data Sheet 1. For all results, a $p < 0.05$ was considered statistically significant. An $r < 0.4$ was considered as weakly correlated, $0.4 < r < 0.7$ as moderately correlated and $r > 0.7$ as strongly correlated.

We used SPSS Statistics to perform a first descriptive analysis in order to obtain a general view of the data included in Supplementary Data Sheet 2 and to highlight the presence of strong outliers in concentration level distribution. SPSS considers data as an outlier if it is outside the following ranges: above the 3rd quartile + 1.5 interquartile range or below the 1st quartile −1.5 interquartile range and indicates it with an asterisk in the graph.

We used Kolmogorov-Smirnov test to check if our data followed a normal distribution and we used Wilcoxon test to check the presence of any differences in metabolites levels among sex.

SPSS Statistics was used to perform partial correlation analyses checked for the effect of chronological age were used to investigate associations between the level of the one-carbon metabolites and cognitive scores.

Chi-squared test with Fisher's exact test was performed to check association between metabolites level considered as high or low compared to 25, 50 and 75 percentiles threshold and QI level considered as high or low compared to the median.

4. Results

4.1 Cognitive profiles in children and adolescents with Down syndrome

4.1.1 Defining the cognitive profile of the sample as a whole

No children performed at the floor for any subtest, while only one child performed at the ceiling only for the Object Assembly subtest. The profile of the sample as a whole was investigated by running two ANOVAs, one with Index as the within-subject factor, the other with Subtest as the within-subject factor. No effect of Index ($p = 1.000$, $\eta p2 < 0.001$, $BF\ 10 = 0.17$) or Subtest emerged ($p\ [gg] = 1.000$, $\eta p2 < 0.001$, $BF\ 10 = 0.006$), suggesting a flat cognitive profile. The standard deviations were high, however, suggesting a marked interindividual variability.

4.1.2 Identifying clusters

Hierarchical cluster analysis using Ward's method and Euclidean distances resulted in the following indices: 9 indices pointed to two as the best number of clusters, while 10 indicated three, and 4 suggested four. Taking the majority rule approach, the best number of clusters was three (C1, C2, C3).

To confirm as much, models with one to four clusters were run on the latent profiles analysis, which revealed that the 3-cluster solution provided the best overall model fit for the data, confirming the assumption of a heterogeneous developmental picture within the sample of children/adolescents with DS that could be identified with the aid of mixture modelling. The results are presented in Table 1.

	Overall model fit			
	1 cluster	2 clusters	3 clusters	4 clusters
BIC	1204.59	1204.15	1204.34	1214.58
Entropy		0.55	0.85	0.87
BLRT (p value)		0.02	0.03	0.28

Table 1. Comparison of overall model fit statistics for latent profiles analysis considering 1–4 clusters*. Onnivello et al. 2022a*

Though the 3-cluster solution did not have the lowest BIC, it did have a better entropy value than the 2-cluster solution. On examining the BLRT findings it emerged that the 3-cluster model showed a better fit than the 2-cluster model, and there was no additional improvement in the fit with the 4-cluster model.

Although residuals were adopted in the analyses, descriptive statistics are reported for AE scores as they are more readily interpretable (Table 2). The three groups of participants were labeled as follows: C1, the Verbal Profile group (scoring higher on verbal than non-verbal index); C2, the Non-Verbal Profile group (scoring higher on non-verbal index); and C3, the Homogeneous Profile group (with similar verbal and non-verbal indices). The three groups were similar in terms of the numbers of participants in each one. No significant differences emerged between the three groups in terms of CA ($p > .05$, $BF10 = 0.12$).

	Verbal profile (n = 29) M (SD)	Non-verbal profile (n = 22) M (SD)	Homogeneous profile (n = 21) M (SD)
Verbal index	55.80 (10.59)	29.36 (11.02)	63.44 (14.20)
Receptive vocabulary	55.64 (17.11)	33.00 (14.57)	62.78 (18.14)
Picture naming	56.37 (13.45)	27.66 (14.36)	64.14 (13.92)
Information	55.38 (14.10)	27.42 (16.07)	63.41 (16.08)
Non-verbal index	41.11 (9.38)	40.11 (11.06)	63.06 (9.06)
Block design	42.77 (15.59)	36.86 (15.48)	64.01 (13.15)
Object assembly	39.45 (8.87)	43.36 (9.56)	62.11 (9.76)
Total index	49.92 (8.47)	33.66 (8.34)	63.28 (11.03)
Chronological age	133.65 (31.17)	134.18 (34.52)	135.57 (29.16)

Table 2. Descriptive statistics for the three clusters (AE scores). *Onnivello et al. 2022a*

4.1.3 Comparison between clusters—verbal and non-verbal indices.

The results of the repeated-measures ANOVA with Index as the within-subject variable are graphically represented in Figure 3. A significant effect of Cluster emerged ($F(2,69) = 89.51$, $p < 0.001$, $\eta p2 = 0.72$, BF 10 = 1.03 × 10 14), and subsequent post-hoc analyses showed that the Homogeneous Profile group's scores were higher than the Verbal Profile group ($t = 7.07$, $p < 0.001$, $d = 0.85$, BF10 = 3.77 × 107) or the Non-Verbal Profile group ($t = 11.81$, $p < 0.001$, $d = 1.57$, BF 10 = 1.72 × 10 16). The Non-Verbal Profile group had lower scores than the Verbal Profile group ($t = -6.20$, $p < 0.001$, $d = 0.83$, BF 10 = 1.52 × 10 7). No main effect of Index was found. The Cluster × Index interaction was significant ($F(2,69) = 26.93$, $p < 0.001$, $\eta p2 = 0.44$ BF10 = 1.14 × 10 23), so post-hoc analyses were run (Table 3). Using Bonferroni's correction, we adjusted the alpha levels to 0.016 (i.e., 0.05/3) for the comparisons between groups, and to 0.025 (i.e., 0.05/2) for the comparisons between Verbal and Non-Verbal Indices within the three groups.

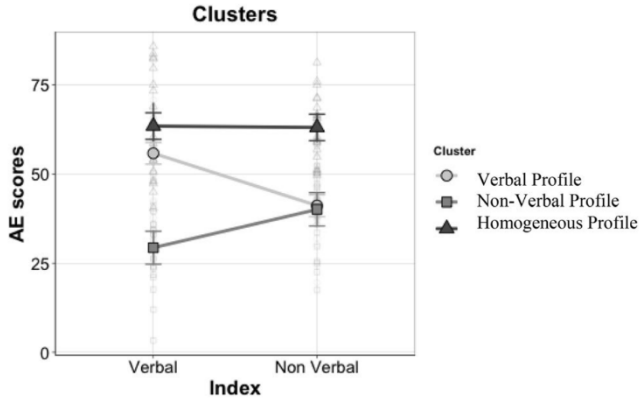

Figure 3. The cognitive profiles based on the indices. *Onnivello et al. 2022a*

	Between-subjects comparison			Within-subject comparison		
	Verbal profile vs non-verbal profile	Verbal profile vs homogeneous profile	Non-verbal profile vs homogeneous profile	Verbal profile	Non-verbal profile	Homogeneous profile
Verbal index	$t = 10.96$ $p < .001$ $d = 1.19$ $BF_{10} = 9.53 \times 10^{13}$	$t = -2.29$ $p = 0.03$ $d = 0.32$ $BF_{10} = 3.17$	$t = -9.88$ $p < .001$ $d = 1.40$ $BF_{10} = 3.03 \times 10^{19}$	$t = 5.83$ $p < .001$ $d = 0.61$ $BF_{10} = 6.69 \times 10^{3}$	$t = -4.36$ $p < .001$ $d = 0.60$ $BF_{10} = 1.12 \times 10^{2}$	$t = -0.94$ $p = 0.36$ $d = 0.10$ $BF_{10} = 0.34$
Non-verbal index	$t = 0.41$ $p = 0.70$ $d = 0.05$ $BF_{10} = 0.30$	$t = -8.13$ $p = < .001$ $d = 0.96$ $BF_{10} = 2.89 \times 10^{10}$	$t = -10.44$ $p < .001$ $d = 0.94$ $BF_{10} = 2.89 \times 10^{7}$			

Table 3. Post-hoc analyses*, cluster × index. t t-test value; p significance level; d Cohen's d expressing the effect size; BF10 Bayes factor expressing the probability of the data given H1 relative to H0. Onnivello et al. 2022a*

Considering the within-subject comparisons, the Verbal Profile group had significantly higher scores in the Verbal Index (M = 55.80) than in the Non-Verbal Index (M = 41.11); vice versa,

the Non-Verbal Profile group scored significantly higher in the Non-Verbal Index (M = 40.11) than in the Verbal Index (M = 29.36); and for the Homogeneous Profile group there was no significant difference between the Verbal and Non-Verbal Indices (M = 63.44 and M = 63.06, respectively).

The between-subjects comparisons showed that: the Verbal Profile group scored significantly higher than the Non-Verbal Profile group in the Verbal Index (M = 55.80 and M = 29.36, respectively); the Non-Verbal Profile group scored significantly lower than the Homogeneous Profile group in both the Verbal Index (M = 63.44 and M = 29.36, respectively) and the Non-Verbal Index (M = 63.06 and M = 40.11, respectively); and the Homogeneous Profile group scored higher scores than the Verbal Profile group in the Non-Verbal Index (M = 63.06 and M = 41.11, respectively).

4.1.4 Comparison between clusters—WPPSI subtests.

The second repeated-measures ANOVA was run to examine the profiles by single subtest. The results are graphically represented in Figure 4.

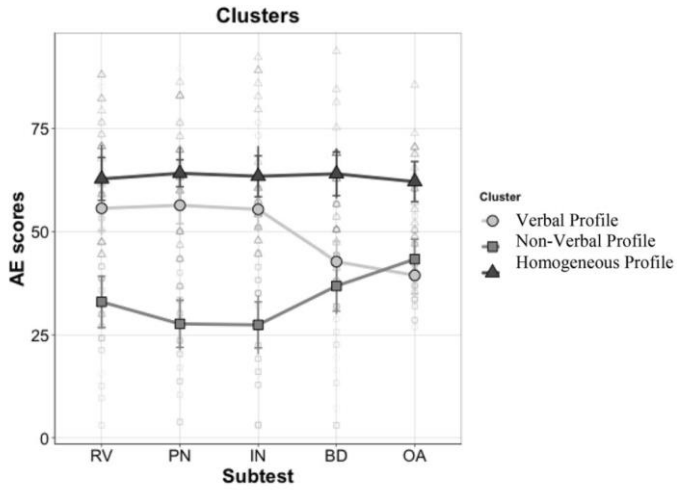

Figure 4. The cognitive profiles based on the subtests*. RV = Receptive Vocabulary, PN = Picture Naming, IN= Information, BD = Block Design, OA = Object Assembly. Onnivello et al. 2022a*

There was an effect of Cluster ($F(2,69) = 90.03$, $p < 0.001$, $\eta p2 = 0.72$, BF 10 = 1.68×10 17), and subsequent post-hoc analyses showed that the Homogeneous Profile group scored higher than the Verbal Profile group ($t = 7.64$, $p < 0.001$, $d = 0.75$, BF 10 = 1.95×10 14) or the Non-Verbal Profile group ($t = 15.44$, $p < 0.001$, $d = 1.58$, BF 10 = 8.87×10 32). The Verbal Profile

group scored higher than the Non-Verbal Profile group (t = 9.02, p < 0.001, d = 0.95, BF 10 = 9.74 × 10 9). There was no main effect of Subtest. A Cluster x Subtest interaction emerged (F(8,276) = 9.58, p < 0.001, η p2 = 0.21, BF 10 = 1.53 × 10 9), so post-hoc analyses were run (see Table 4 for between- subjects comparisons, and Table 5 for within-subject comparisons). Using Bonferroni's correction, we adjusted the alpha levels to 0.016 (i.e., 0.05/3) for comparisons between groups, and to 0.005 (i.e., 0.05/10) for comparisons between subtests within the three groups.

	Between-subjects comparison		
	Verbal profile vs non-verbal profile	**Verbal profile vs homogeneous profile**	**Non-verbal profile vs homogeneous profile**
Verbal index			
Receptive Vocabulary	t = 5.99 p < .001 d = 0.73 $BF_{10} = 3.70 \times 10^4$	t = −1.48 p = 0.15 d = 0.21 $BF_{10} = 0.73$	t = −6.45 p < .001 d = 0.88 $BF_{10} = 1.16 \times 10^5$
Picture Naming	t = 8.11 p < .001 d = 0.93 $BF_{10} = 9.35 \times 10^7$	t = −2.10 p = 0.04 d = 0.24 $BF_{10} = 1.80$	t = −9.04 p < .001 d = 1.07 $BF_{10} = 2.22 \times 10^8$
Information	t = 7.01 p < .001 d = 0.91 $BF_{10} = 1.82 \times 10^6$	t = −1.84 p = 0.07 d = 0.25 $BF_{10} = 1.20$	t = −7.83 p < .001 d = 1.07 $BF_{10} = 6.53 \times 10^6$
Non-verbal index			
Block Design	t = 1.46 p = 0.15 d = 0.18 $BF_{10} = 0.69$	t = −5.95 p < .001 d = 0.68 $BF_{10} = 2.29 \times 10^4$	t = −6.54 p < .001 d = 0.81 $BF_{10} = 1.18 \times 10^5$
Object Assembly	t = −1.50 p = 0.14 d = 0.13 $BF_{10} = 0.71$	t = −9.25 p < .001 d = 0.72 $BF_{10} = 9.60 \times 10^8$	t = −7.10 p < .001 d = 0.56 $BF_{10} = 6.98 \times 105$

Table 4. Post-hoc analyses*, subtest × index—between-subjects comparison—groups paired comparisons in each subtest. t t-test value; p significance level; d Cohen's d expressing the effect size; BF10 Bayes factor expressing the probability of the data given H1 relative to H0. Onnivello et al. 2022a*

		Within-subject comparison		
		Verbal profile	**Non-verbal profile**	**Homogeneous profile**
Receptive Vocabulary	Picture Naming	t = −0.50 p = 0.62 d = 0.06 BF_{10} = 0.22	t = 1.11 p = 0.28 d = 0.14 BF_{10} = 0.39	t = −0.77 p = 0.45 d = 0.08 BF_{10} = 0.30
	Information	t = −0.36 p = 0.72 d = 0.05 BF_{10} = 0.21	t = 0.99 p = 0.33 d = 0.13 BF_{10} = 0.36	t = −0.78 p = 0.44 d = 0.08 BF_{10} = 0.30
	Block Design	t = 2.48 p = 0.02 d = 0.36 BF_{10} = 2.60	t = −1.76 p = 0.09 d = 0.25 BF_{10} = 0.83	t = −1.36 p = 0.19 d = 0.16 BF_{10} = 0.51
	Object Assembly	t = 4.32 p < .001 d = 0.48 $BF_{10} = 1.56 \times 10^2$	t = −3.62 p = 0.001 d = 0.45 BF_{10} = 23.74	t = −0.81 p = 0.43 d = 0.10 BF_{10} = 0.30
Picture Naming	Information	t = 0.10 p = 0.92 d = 0.01 BF_{10} = 0.20	t = −0.13 p = 0.90 d = 0.01 BF_{10} = 0.22	t = 0.005 p = 1.00 d = 0.001 BF_{10} = 0.23
	Block Design	t = 3.59 p = 0.001 d = 0.42 BF_{10} = 27.41	t = −2.55 p = 0.02 d = 0.39 BF_{10} = 2.97	t = −1.03 p = 0.31 d = 0.08 BF_{10} = 0.36
	Object Assembly	t = 4.52 p < .001 d = 0.54 $BF_{10} = 2.55 \times 10^2$	t = −5.39 p < .001 d = 0.60 $BF_{10} = 9.96 \times 10^2$	t = −0.23 p = 0.82 d = 0.02 BF_{10} = 0.24
Information	Block Design	t = 3.26 p = 0.003 d = 0.41 BF_{10} = 13.19	t = −2.82 p = 0.01 d = 0.39 BF_{10} = 4.89	t = −0.61 p = 0.55 d = 0.06 BF_{10} = 0.27
	Object Assembly	t = 4.79 p < .001 d = 0.53 $BF_{10} = 4.97 \times 10^2$	t = −4.53 p < .001 d = 1.46 $BF_{10} = 1.61 \times 10^2$	t = −0.21 p = 0.83 d = 0.02 BF_{10} = 0.23
Block design	Object Assembly	t = 1.17 p = 0.25 d = 0.11 BF_{10} = 0.36	t = −2.24 p = 0.04 d = 0.21 BF_{10} = 1.73	t = 0.59 p = 0.56 d = 0.06 BF_{10} = 0.27

***Table 5. Post-hoc analyses**, subtest × index—within-subject comparisons. Paired comparisons between subtests within each group. t t-test value; p significance level; d Cohen's d expressing the effect size; BF10 Bayes factor expressing the probability of the data given H1 relative to H0. Onnivello et al. 2022a*

Between-subjects comparisons showed that the Verbal Profile group scored higher than the Non-Verbal Profile group in all the subtests contributing to the Verbal Index. No differences emerged for the Non-Verbal Index subtests. The Verbal Profile group had lower scores than the Homogeneous Profile group in all the Non-Verbal Index subtests, while the Non-Verbal Profile group scored lower than the Homogeneous Profile group in the subtests contributing to both indices.

In the within-subject comparisons, the Verbal Profile group scored lower on Object Assembly than in any of the other Verbal Index subtests, and lower on Block Design than on Picture Naming or Information. The Non-Verbal Profile group scored higher on Object Assembly than on any of the other Verbal Index subtests. The Homogeneous Profile group showed no significant differences between the subtests.

4.1.5 The role of medical problems

Considering medical problems, the percentages of individuals with heart problems, a history of heart surgery and OSA are given in Table 6. The chi-squared test revealed no associations between any of these medical conditions and group ($p < 0.05$).

	Verbal (N = 29)	Non-verbal (N = 22)	Homogeneous (N = 21)	N condition/N whole sample[a]
Heart problems % yes (n)	37% (15)	30% (12)	32% (13)	40/64
Prior heart surgery % yes (n)	30% (6)	40% (8)	30% (6)	20/63
OSA % yes (n)	29% (5)	29% (5)	42% (7)	17/56

Table 6. Prevalence of medical conditions in each group. *a Data on the presence of these conditions were not available for some participants. Onnivello et al. 2022a*

4.1.6 The role of mothers' education

	Verbal (N = 29)	Non-verbal (N = 22)	Homogeneous (N = 21)	N condition/N whole sample
Mothers' education > high school % (n)	43% (14)	27% (9)	30% (10)	33/72
Fathers' education > high school % (n)	42%(11)	27% (7)	31% (8)	26/72

Table 7. Prevalence of parents with an education level higher than high school. *Onnivello et al. 2022a*

Table 7 shows the percentage of mothers with the different education levels in each subgroup. A chi-squared test exploring the association between parents' education levels and group revealed no association between the mothers' education level and the groups. Mothers' and fathers' education levels (coded as 1 = primary school, 2 = middle school, 3 = high school, and 4 = university) were then correlated with the WPPSI global score. A positive and moderate

correlation emerged in the Non-Verbal Profile between the mothers' education levels and the WPPSI global score. The correlations are given in Table 8.

	Verbal	Non-verbal	Homogeneous
Mothers' education	0.130	0.307	0.060
Fathers' education	0.042	0.283	0.137

Table 8. Correlations between parents' education levels and WPPSI global score by group. *Onnivello et al. 2022a*

4.1.7 Developmental trajectories

The descriptive statistics show that the Non-Verbal Profile group reached each milestone later than the other two groups, although the difference was only significant for babbling ($F(2,57) = 4.22$, $p = 0.02$, $\eta p2 = 0.13$), where the Non-Verbal Profile group reached this milestone later than the Homogeneous Profile group ($t = 2.89$, $p = 0.02$, $d = 0.81$). The age of reaching all milestones were then correlated with the total WPPSI score: sitting, babbling, walking, and first words correlated negatively and moderately with the global score in the Non-Verbal Profile group. All the correlations are given in Table 9.

	Verbal	Non-verbal	Homogeneous
Sitting	0.028	−0.652**	0.035
Babbling	−0.151	−0.675**	−0.233
Walking	−0.222	−0.572*	0.060
First words	−0.242	−0.385	−0.108

Table 9. Correlations between age of reaching developmental milestones and WPPSI global score in each group. *** < .001, ** < .01, * < .05. *Onnivello et al. 2022a*

4.2 Executive functions and adaptive behaviour in individuals with Down syndrome

4.2.1 Executive functions

Executive functions in the preschooler group

Among the children with DS aged 3–6.11 years, almost half of the sample had clinically elevated T-scores for Working Memory and the Emergent Metacognition Index (47.5% and 40%, respectively). In contrast, only 5% of the sample had clinically elevated T-scores for Emotional Control and the Flexibility Index.

T-scores obtained from the Global Executive Composite scores were significantly above the norm of 50. Of the three indexes, however, only the Emergent Metacognition Index was significantly above the norm, suggesting that the main EF deficit in DS at this age concerns Emergent Metacognition. T-scores for the five scales were also compared with the norm, using a Bonferroni correction (α 0.05/5 = 0.01). Some subscales indicated significant difficulties, suggesting a profile of strengths and weaknesses, the former in Emotional Control and the latter in Inhibit, Working Memory and Plan/Organise. Then, the EF profiles were investigated by running two different repeated-measures ANOVAs. In one, the three indexes were dependent variables and Index was the within-group variable. In the other, the seven scales were dependent variables, and Scale was the within-group variable. T-scores were considered for both analyses. A significant effect of Index emerged ($F_{2,78} = 44.21$, $P < 0.001$, $\eta p\ 2 = 0.53$), and subsequent post-hoc analyses showed higher scores in Emergent Metacognition than in Inhibitory Self-Control (Mdiff. = 12.13, $P < 0.001$, $d = 1.01$) or Flexibility (Mdiff. = 14.83, $P < 0.001$, $d = 1.46$). There was also a significant effect of Scale, $F_{2.87,112.02} = 29.70$, Pgg < 0.001, $\eta p\ 2 = 0.43$, and post-hoc analyses identified significant differences between Emotional Control, which had the lowest score (and was therefore the greatest strength), and all the other scales: Inhibit (Mdiff. = -9.15, $P < 0.001$, $d = 0.93$), Shift (Mdiff. = -5.96, $P = 0.014$, $d = 0.54$), Working Memory (Mdiff. = -19.63, $P < 0.001$, $d = 1.75$) and Plan/Organise (Mdiff. = -11.15, $P < 0.001$, $d = 1.18$). Working Memory, which had the highest score (making it the greatest weakness), differed significantly from Inhibit (Mdiff. = 10.47, $P < 0.001$, $d = 0.80$), Shift (Mdiff. = 13.65, $P < 0.001$, $d = 1.09$) and Plan/Organise (Mdiff. = 8.47, $P < 0.001$, $d = 1.05$).

Figure 5 shows the profiles, considering both the indexes and the scales.

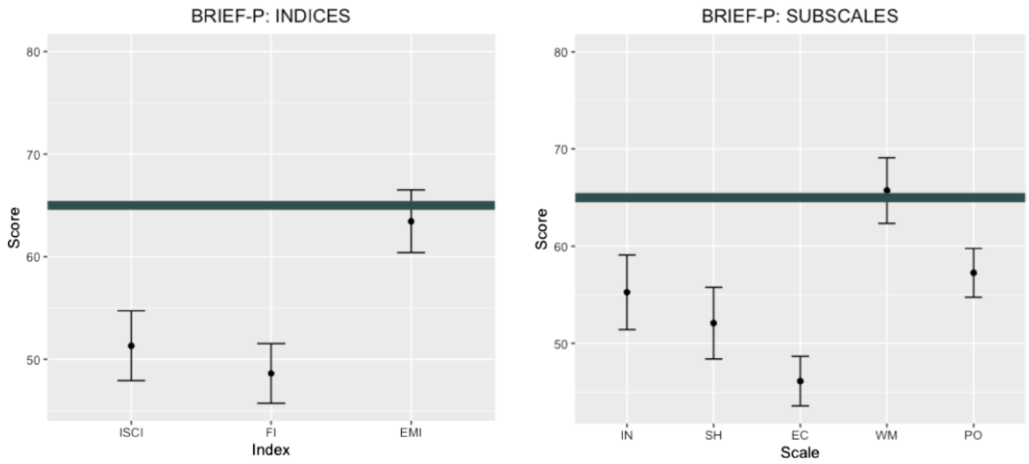

Figure 5. BRIEF-P scores in the preschooler group. *BRIEF-P, Behaviour Rating Inventory of Executive Function – Preschool Version; EMI, Emergent Metacognition Index; FI, Flexibility Index; IN, Initiate; INH, Inhibit; ISCI, Inhibitory Self-Control Index; PO, Plan/Organise; SH, Shift; WM, Working Memory. The thick line represents the BRIEF-P cut-off of 65. Scores higher than this value are in the clinically elevated range. [Colour figure can be viewed at wileyonlinelibrary.com]. Figure by Onnivello et al. 2022b.*

Executive functions in the school-age group

For children with DS aged 7–16 years, the highest percentages of clinically elevated scores emerged for Shift (37%) and Task-Monitor (32%), and the lowest percentages being for Emotional Control (10%) and Organisation of Materials (12%).

T-scores obtained from the Global Executive Composite scores were significantly above the norm of 50, and so were those for the three indexes concerning Behaviour Regulation, Emotional Regulation and Cognitive Regulation. The T-scores for each of the nine scales were also compared with the norm of 50, using Bonferroni's correction (α 0.05/9 = 0.006). The scores were significantly higher than 50 for almost all the scales (Inhibit, Self-Monitor, Shift, Initiate, Working Memory, Plan/Organise and Task-Monitor), but not for Emotional Control or Organisation of Materials. Here again, the EF profiles were investigated by running two different repeated-measures ANOVAs. In one, the three indexes were dependent variables, and Index was the within-group variable. In the other, the nine scales were dependent variables, and Scale was the within-group variable. T-scores were considered for both analyses.

A significant effect of Index emerged, $F_{2,118} = 13.40$, $P < 0.001$, $\eta p\ 2 = 0.19$, and subsequent post-hoc analyses showed higher scores in the Cognitive Regulation Index than in the Behaviour Regulation Index (Mdiff. = 4.15, $P < 0.001$, $d = 0.59$) or Emotion Regulation Index

(Mdiff. = 5.07, P < 0.001, d = 0.59). There was also a significant effect of Scale, F6.39,376.98 = 16.61, Pgg < 0.001, ηp 2 = 0.22. As shown in Fig. 2, Emotional Control and Organisation of Materials scored the lowest, and Shift the highest. Post-hoc analyses identified significant differences between Emotional Control and Inhibit (Mdiff. = -6.52, P < 0.001, d = 0.72), Self-Monitor (Mdiff. = -7.10, P = 0.003, d = 0.55), Shift (Mdiff. = -12.30, P < 0.001, d = 0.92), Initiate (Mdiff. = -10.57, P < 0.001, d = 0.86), Working Memory (Mdiff. = -11.38, P < 0.001, d = 1.15), Plan/Organise (Mdiff. = -11.35, P < 0.001, d = 1.02) and Task-Monitor (Mdiff. = -11.98, P < 0.001, d = 0.97). Organisation of Materials differed significantly from Initiate (Mdiff. = -7.20, P < 0.001, d = 0.61), Working Memory (Mdiff. = -8.07, P < 0.001, d = 0.76), Plan/ Organise (Mdiff. = -8.03, P < 0.001, d = 0.76), Shift (Mdiff. = -8.98, P < 0.001, d = 0.73) and Task-Monitor (Mdiff. = -8.66, P < 0.001, d = 0.71). Shift differed from Inhibit (Mdiff. = 5.78, P = 0.04, d = 0.44). Figure 6 shows the profiles, considering both the indexes and the scales.

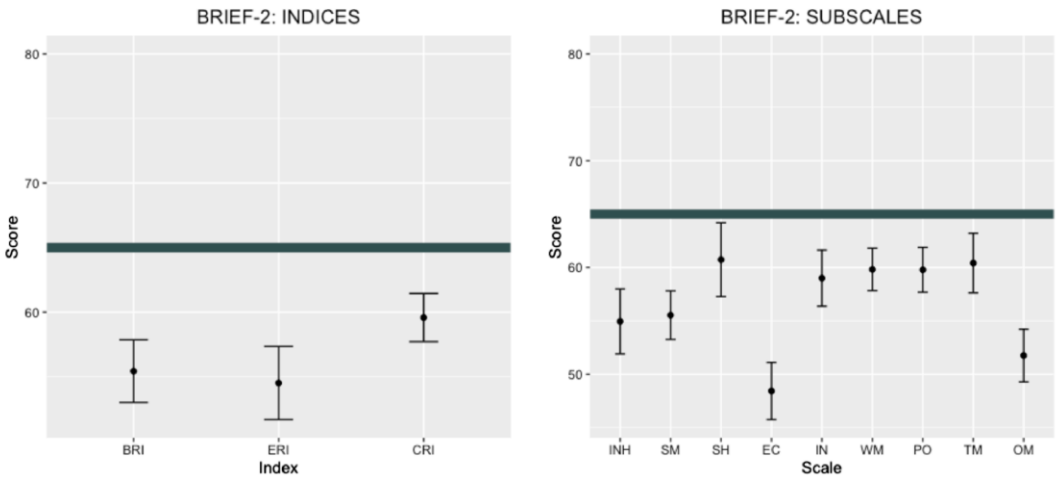

Figure 6. BRIEF-2 scores in the school-age group. *BRI, Behaviour Regulation Index; CRI, Cognitive Regulation Index; BRIEF-P, Behaviour Rating Inventory of Executive Function – Preschool Version; EC, Emotional Control; ERI, Emotion Regulation Index; IN, Initiate; INH, Inhibit; OM, Organisation of Materials; PO, Plan/Organise; SH, Shift; SM, Self-Monitor; TM, Task-Monitor; WM, Working Memory. The thick line represents the BRIEF-P cut-off of 65. Scores higher than this value are in the clinically elevated range. [Colour figure can be viewed at wileyonlinelibrary.com]. Figure by Onnivello et al. 2022b*

Executive functions: profile comparison

BRIEF-P and BRIEF 2 have five scales in common (Inhibit, Shift, Emotional Control, Working Memory and Plan/Organise) and, because of that, the EF profiles of the two groups could be compared with a 5 × 2 ANOVA, with Scale as the within factor and Group as the between factor. The main effect of Scale (F3.38,321.41 = 45.34, Pgg < 0.001, ηp 2 = 0.31) and the ScaleXGroup interaction (F3.38,321.41 = 9.73, Pgg < 0.001, ηp 2 = 0.09) were significant, but the main effect of Group was not. Figure 7 is a graphical representation of the data.

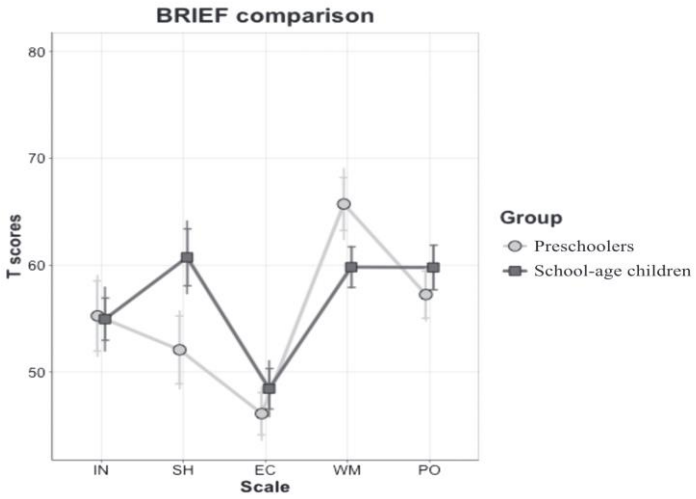

Figure 7. BRIEF profile comparison. *BRIEF, Behaviour Rating Inventory of Executive Function; EC, Emotional Control; INH, Inhibit; PO, Plan/Organise; SH, Shift; WM, Working Memory. Onnivello et al. 2022b*

Between-group comparisons showed a significant difference in Shift, the older group having higher scores. Within-group comparisons indicated that both groups had Emotional Control as a strength and Working Memory as a weakness, while it was only in the school-age group that difficulties also emerged in Shift and Plan/Organise.

4.2.2 Adaptive behaviour

Adaptive behaviour in the preschooler group

Standardised scores obtained from the Adaptive Behaviour Composite scores were significantly below the norm of 100 (Table 10). Among the children with DS aged 3–6.11 years,

far more than half of the sample had clinically low standardised scores on Communication, Daily Living Skills and Motor Skills (85%, 70% and 87.5%, respectively). In contrast, only 20% of the sample showed clinically low standardised scores for Socialisation.

Adaptive behaviour in the school-age group

Standardised scores from the Adaptive Behaviour Composite scores were significantly below the norm of 100 (Table 10). Among these older children with DS, most of the sample had clinically low standardised scores in all three domains: Communication, Daily Living Skills and Socialisation (85%, 86.7% and 70%, respectively).

	%CE[†]	Mean	SD	t[‡]	*P* value	Cohen's *d*
Preschoolers						
Communication	85	55.75	14.07	−19.88	<0.001	3.14
Daily Living Skills	70	62.28	13.00	−18.35	<0.001	2.90
Socialisation	22.5	74.58	13.23	−12.15	<0.001	1.92
Motor Skills	87.5	55.70	12.78	−21.93	<0.001	3.48
Adaptive Behaviour Composite score	82.5	55.73	11.96	−23.41	<0.001	3.70
School-age children						
Communication	85	49.72	18.83	−20.69	<0.001	2.67
Daily Living Skills	86.7	50.22	18.82	−20.48	<0.001	2.65
Socialisation	70	60.60	17.45	−17.49	<0.001	2.26
Adaptive Behaviour Composite score	78.6	46.91	18.46	−27.26	<0.001	2.87

[†]Percentage of individuals with DS reportedly in the low range ($T < 70$).
[‡]Comparison with normative standardised score of 100.

Table 10. ***Percentages of clinically high standard scores, means, standard deviations (SDs) and one-sample t-test results on the Vineland Adaptive Behaviour Scales, Second Edition.*** *Onnivello et al. 2022b*

Adaptive behaviour: group comparison

The two age groups were compared using a repeated-measures ANOVA, where Scale was the within factor and Group the between factor (refer to Figure 8 for a graphical representation). The main effects of Scale ($F_{2,196} = 59.00$, $P < 0.001$, $\eta p\ 2 = 0.37$) and Group were significant ($F_{1,98} = 13.06$, $P < 0.001$, $\eta p\ 2 = 0.12$), and so was the ScaleXGroup interaction ($F_{2,196} = 4.20$, $P = 0.016$, $\eta p\ 2 = 0.04$).

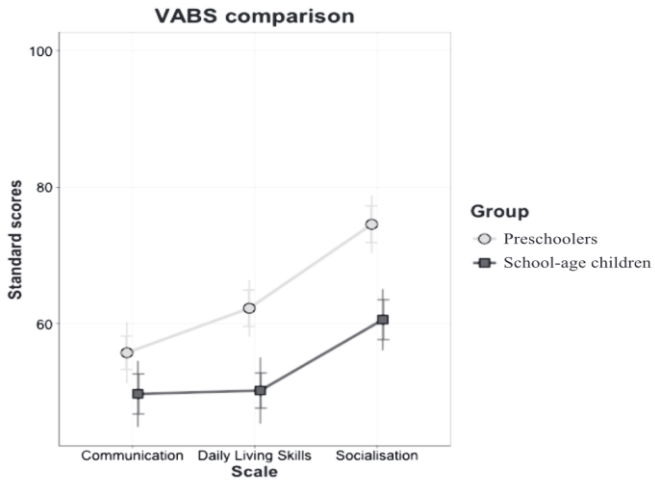

Figure 8. ***VABS profile comparison. VABS, Vineland Adaptive Behaviour Scales.*** *Onnivello et al. 2022b*

Post-hoc analyses on the effect of Scale showed that Socialisation was the highest score, and it differed significantly from Communication (Mdiff. = 14.06, P < 0.001, d = 0.93) and Daily Living Skills (Mdiff. = 11.15, P < 0.001, d = 0.81). Based on Bonferroni's correction, the alpha levels were adjusted to 0.016 (i.e. 0.05/3) for the comparisons between the groups on each scale, and to 0.008 (i.e. 0.05/6) for comparisons between the scales within each group. Between-group comparisons indicated significant differences in Daily Living Skills and Socialisation, with the younger group obtaining higher standardised scores. Within-group comparisons showed a similar picture for the two groups, with Communication and Daily Living Skills differing significantly from Socialisation, which emerged as a relative strength.

4.2.3 Executive functions and adaptive behaviour

Correlations between VABS-II and BRIEF-P/BRIEF 2

The relationship between the VABS-II scales and the BRIEF-P and BRIEF 2 indexes was investigated. In the younger group, the only significant (r=-0.33, p<0.05) correlation that came to light was between the Emergent Metacognition Index and Communication. This correlation is explained mainly by the significant correlation between Working Memory and Communication. In the older group (Table 11), on the other hand, all three indexes (Behaviour Regulation, Emotion Regulation and Cognitive Regulation), and almost all the scales

correlated significantly with the three adaptive behaviour indexes (Communication, Daily Living Skills and Socialisation).

	Communication	Daily Living Skills	Socialisation
Index			
Behaviour Regulation Index[†]	−0.48***	−0.47***	−0.49***
Emotion Regulation Index[‡]	−0.41***	−0.46***	−0.50***
Cognitive Regulation Index[§]	−0.50***	−0.43***	−0.45**
Subscale			
Inhibit	−0.41***	−0.45***	−0.41***
Self-Monitor	−0.31*	−0.20	−0.27*
Shift	−0.34**	−0.38**	−0.47***
Emotional Control	−0.33**	−0.37**	−0.34**
Initiate	−0.39**	−0.38**	−0.31**
Working Memory	−0.55***	−0.39**	−0.33**
Plan/Organise	−0.27*	−0.29*	−0.31**
Task-Monitor	−0.34**	−0.19	−0.33**
Organisation of Materials	−0.28*	−0.36**	−0.36**

BRIEF, Behaviour Rating Inventory of Executive Function; VABS, Vineland Adaptive Behaviour Scales.
[†]Behaviour Regulation = Inhibit + Self-Monitor.
[‡]Emotion Regulation = Shift + Emotional Control.
[§]Cognitive Regulation = Initiate + Working Memory + Plan/Organise + Organisation of Materials + Task-Monitor.

Table 11. Correlations between BRIEF 2 and VABS in the school-age group. *Onnivello et al. 2022b*

Regression analyses

Simple linear regressions were run for both groups to examine the role of EFs (BRIEF-P and BRIEF 2) on the children's adaptive behaviour (Vineland-II). For each model, age was entered first, followed by the indexes (ISCI, FI and EMI for BRIEF-P, and BRI, ERI and CRI for BRIEF 2), while each Vineland-II scale (Communication, Daily Living Skills and Socialisation) was the outcome variable. When an index emerged as a predictor, further analyses were run to detect which scale of the index was most predictive.

For the younger group, of all the EF indexes and adaptive behaviour scores, the Emergent Metacognition Index was the only significant predictor (β = -0.33, P = 0.04) for Communication. Within the subscales comprising this index (Working Memory and Plan/Organise), Working Memory emerged as the significant predictor (β = -0.34, P = 0.03). No significant predictors were identified for Socialisation or Daily Living Skills.

For the older group, the analyses showed that the Cognitive Regulation Index was a predictor of Communication (β = -0.33, P = 0.04). In subsequent analyses on the scales comprising this

index, Working Memory emerged as the significant predictor (β = -0.55, P < 0.001). The Behaviour Regulation Index and age were predictors of Daily Living Skills (β = -0.46, P < 0.001, β = -0.26, P = 0.03 respectively), and the Inhibit scale was the significant predictor (β = -0.45, P < 0.001). Two indexes, Behaviour Regulation and Emotion Regulation, together with age, were predictive of Socialisation (β = -0.31, P = 0.01, β = -0.27, P = 0.03, β = -0.49, P < .001 respectively). Then, when the single scales were considered, Inhibit was identified as the significant predictor (β = -0.41, P = 0.001), together with Shift (β = -0.47,P < 0.001).

4.3 Cross-sectional developmental trajectories in the adaptive functioning of children and adolescents with Down syndrome

4.3.1 Adaptive functioning as a function of age. Standard scores

AF-ST scores by CA were examined using regression models with null, linear, and segmented relationships. Considering Communication, only the linear model was significant, and it had a better AIC value (980.52, F1, 113 =7.14, p = 0.008, R2 = 0.05) than the segmented model (AIC = 984.28, F2, 111 =0.11, p = 0.89, R2 = 0.03) and the null model (AIC = 985.67). The linear model was significant also for Daily Living Skills scores, with a better AIC value (964.26, F1, 113 =18.48, p < .001, R2 =0.13). Therefore, this model fit better than the segmented model (AIC = 965.23, F2, 111 =1.48, p = 0.23, R2 = 0.13) and the null model (AIC = 979.55). Finally, we found the linear model as the best one also for, Socialization (AIC = 935.85, F1, 113 =46.95, p < .001, R2 = 0.30). The model was significant, and the AIC value was the best one compared to the others. Therefore, the model fit better than the segmented model (AIC = 933.81, F2, 111 =2.99, p = 0.05, R2 = 0.28) and the null model (AIC = 972.66). The linear model was consequently retained as the best model for all three domains. The models for AF-ST scores in relation to CA are represented in Figure 9 (see Fig. 9A for Communication, Fig. 9B for Daily Living Skills, and Fig. 9C for Socialization). In all three cases, standard scores decreased linearly when age increased.

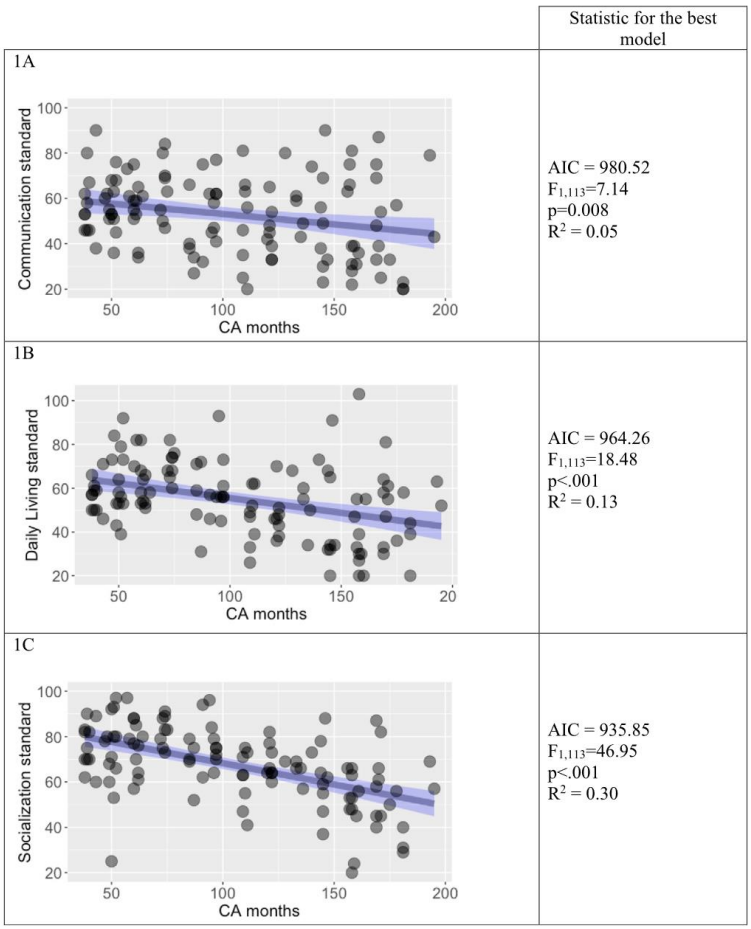

Figure 9. ***Regression models best explaining progression in AF standard scores in relation to CA.*** *(A) Linear model, (B) linear model, and (C) linear model. Onnivello et al. 2024*

4.3.2 Adaptive functioning as a function of chronological age. Age-equivalent scores

The AF-AE scores as a function of CA were examined using regression models with null, linear, and segmented relationships. The linear model provided the best explanation for the Communication scores (AIC = 981.37, F1, 113 =111.44, p < 0.001, R2 = 0.49). This model was significant, and its AIC was better than the segmented model (AIC = 983.69, F2, 111 =0.81, p = 0.44, R2 = 0.49) and the null model (AIC = 1058.48).

A different model emerged as the most suitable for explaining the data concerning Daily Living Skills. Both the linear (AIC = 937.88, F1, 113 =3.50, p = 0.03, R2 = 0.65) and the segmented

(AIC = 934.78, F2, 111 =226.40, $p < 0.001$, R2 = 0.67) models resulted significant, but the second showed a better AIC than the first. The null model had the highest AIC value (1059.02). Therefore, the segmented model was retained as the best one. The breakpoint was estimated at age 75 months. The estimated relationship before the breakpoint was B = 0.75, SE = 0.11, $p < 0.001$, R2 = 0.50, and after the breakpoint it was B = 0.33, SE = 0.06, $p < 0.001$, R2 = 0.29. The segmented model was the most suitable also for Socialization scores. Both the linear (AIC = 953.84, F1, 113 =125.79, $p < 0.001$, R2 = 0.51) and the segmented (AIC = 951.87, F2, 111 =226.40, $p < 0.001$, R2 = 0.53) models were significant, but the best AIC was observed in the segmented model. The null model had the highest AIC value (1035.84). The breakpoint was estimated at age 80.46 months. The estimated relationship before the breakpoint was B = 0.62, SE = 0.14, $p < 0.001$, R2 = 0.32, and after the breakpoint it was B = 0.23, SE = 0.06, $p < 0.001$, R2 = 0.15.

To sum up, the linear model was the best one for Communication, while the segmented model was best for Daily Living Skills and Socialization. In both cases, after, respectively, the age of 75 and 80.46 months, the rhythm of growth of AF tends to decrease. These models for AF-AE scores in relation to CA are graphically represented in Figure 10 (see Fig. 10A for Communication, Fig. 10B for Daily Living Skills, and Fig. 10C for Socialization).

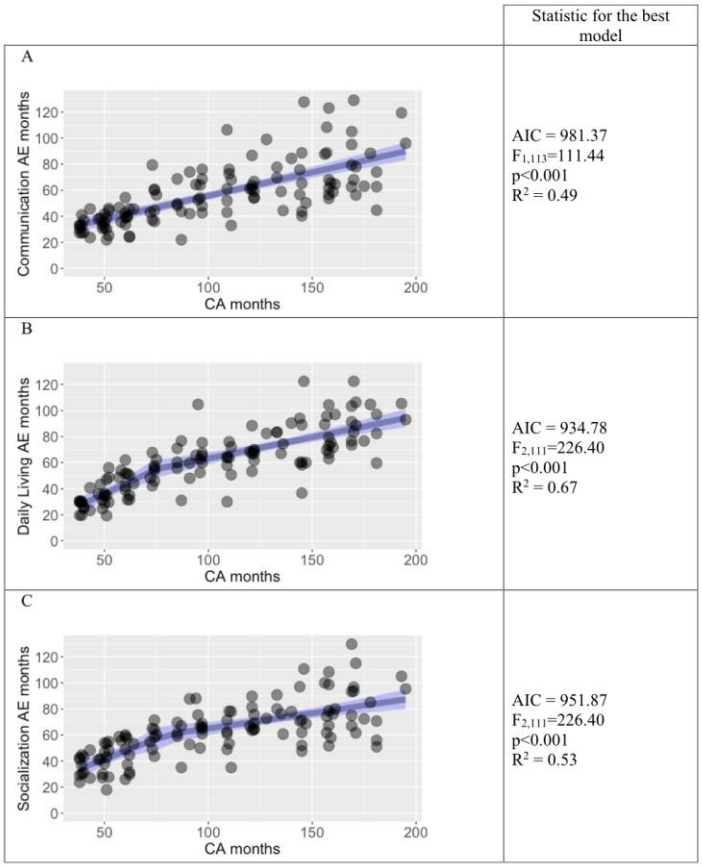

Figure 10. ***Regression models best explaining progression in AF age equivalent scores in relation to CA****. (A) Linear model, (B) segmented model, and (C) segmented model. Onnivello et al. 2024*

4.3.3 Adaptive functioning as a function of mental age. Age-equivalent scores

When the AF-AE scores were considered as a function of MA, the linear model provided the best explanation for the Communication scores (AIC = 916.83, F1, 113 =279.99, p < 0.001, R2 = 0.71). This model was significant and fit better than the null model (AIC = 1058.48) or the segmented model (AIC = 919.21, F2, 111 =0.78, p = 0.46, R2 = 0.71).

The linear model was also the most suitable for Daily living Skills scores (AIC = 933.92, F1, 113 =233.60, p < .001, R2 = 0.67). It was significant, and the AIC was lower in comparison to the null model (AIC = 1059.02) and the segmented model (AIC = 933.20, F2, 111 =2.33, p = 0.10, R2 = 0.67).

Finally, the segmented model best explained the Socialization scores (AIC = 944.03, F2, 111 =4.37, p < 0.001, R2 = 0.56). The model was significant like the linear one, which, however, had a higher AIC value (948.76, F1, 113 =140.08, p < 0.001, R2 = 0.53). The null model showed a higher AIC (AIC = 1035.84). The breakpoint was estimated at age 39.42 months. The estimated relationship before the breakpoint was B = 0.25, SE = 0.04, p < 0.001, R2 = 0.38, and after the breakpoint it was B = 0.23, SE = 0.07, p = 0.003, R2 = 0.15.

In short, the linear model was the best model for Communication and Daily Living Skills, while the segmented model, with a decrease of the rhythm of growth after 39.42 months, was best for Socialization. The models for AF-AE scores in relation to MA are graphically represented in Figure 11 (see Fig. 11A for Communication, Fig. 11B for Daily Living Skills, and 11C for Socialization).

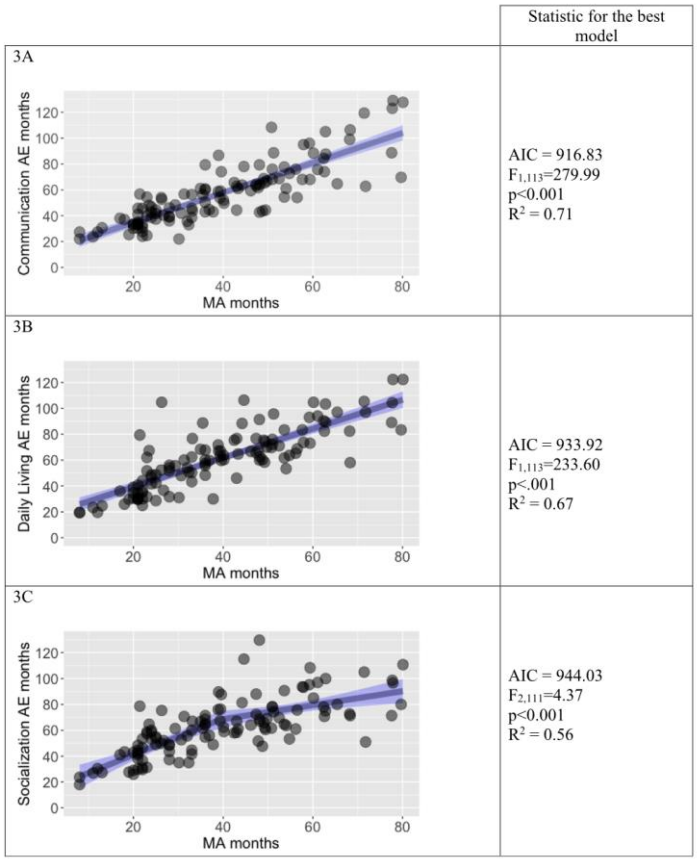

Figure 11. ***Regression models best explaining progression in AF age equivalent scores in relation to MA****. (A) Linear model, (B) linear model and (C) segmented model. Onnivello et al. 2024*

4.3.4 The role of CA and MA

Stepwise regressions were run to explore which variable —CA or MA— better explained the AF-AE scores. The results are shown in Table 12. The model with both CA and MA was the best model for all three domains considered. Together, CA and MA always explained more than half of the variance (R2 = 0.59–0.75). Considering the domain of Communication, the ß was higher for MA than for CA, while similar ß values emerged in the Daily Living Skills and Socialization domains.

Domain AE scores	Best model	Statistics	Standardized coefficient
Communication	CA+MA	$F_{(2,\ 112)}= 145.8$, $p < 0.001$, $R^2 = 0.72$	CA ß= 0.15 ($p = 0.05$) MA ß= 0.73 ($p < 0.001$)
Daily Living Skills	CA+MA	$F_{(2,\ 112)}= 170.9$, $p < 0.001$, $R^2 = 0.75$	CA ß= 0.45 ($p < 0.001$) MA ß= 0.48 ($p < 0.001$)
Socialization	CA+MA	$F_{(2,\ 112)}= 84.29$, $p < 0.001$, $R^2 = 0.59$	CA ß= 0.38 ($p < 0.001$) MA ß= 0.44 ($p < 0.001$)

Table 12. Best Model to Explain AF-AE Scores. *Onnivello et al. 2024*

4.3.5 Comparison between AF and MA as a function of CA

To visually compare the developmental trajectory of AF and MA, we first examined MA as a function of age. With MA as the dependent variable and CA as the independent variable, we tested the null, linear, and segmented models. Both the linear (AIC = 888.93, F2, 111 =168.24, p < 0.001, R2 = 0.56) and segmented (AIC = 881.79, F3, 111 =5.65, p = 0.004, R2 = 0.61) models resulted significant, but the former had a higher AIC value. The null model showed the highest AIC value of the three models (AIC=986.44).

Consequently, MA data were best explained by the segmented model, with 128 months estimated as the breakup point. The estimated relationship before the breakpoint was B = 0.39, SE = 0.03, p < 0.001, R2 = 0.66, and after the breakpoint it was B = 0.09, SE = 0.15, p = 0.56, R2 < 0.001. Then, AF-AE scores and MA scores were plotted as a function of CA. See Figure 12.

Upon visual examination of the trajectories, it became evident that, in respect to CA, AF-AE scores tended to be higher than MA scores in all AF domains. Furthermore, it was observed that the MA trajectory differed from the AF trajectories. In fact, while the MA trajectory tended to flatten after 128 months, Communication, Daily Living Skills, and Socialization scores

continued to increase at later ages, notwithstanding the change in speed around 75 and 80 months, respectively.

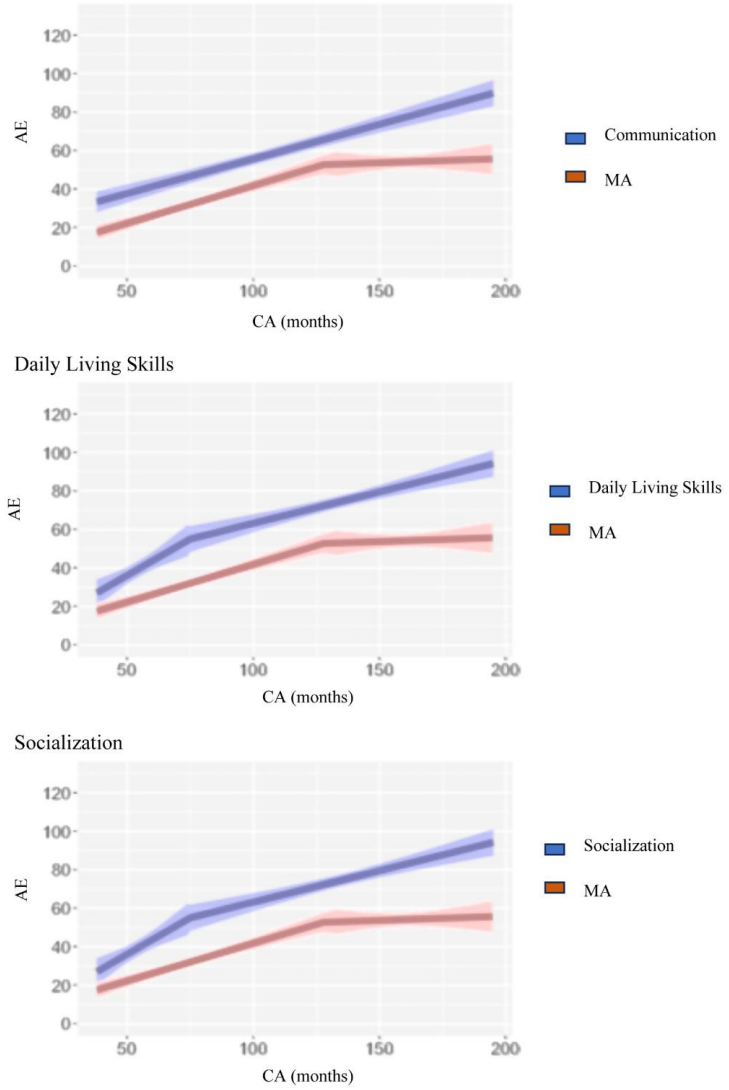

Figure 12. MA and AF AE scores trajectories in relation to CA. *Onnivello et al. 2024*

4.4 Machine learning based analysis for intellectual disability in Down syndrome

The clinical dataset includes a total of 106 DS subjects (41 females; 65 males) for which cognitive data are available. The mean age of cognitive assessment is 8.88 years with a standard deviation (SD) of 3.96 years. Anonymized personal, genetic, diagnostic, clinical, and auxological information are available in the Supplementary Table 1 of Baldo et al. work (2023).

In this section, we are presenting the results of the analysis on the complete dataset, except for the features removed during the preprocessing phase. All the main findings will be presented outlining advantages and disadvantages of each approach motivating the use of the methodologies presented in Materials and Methods section. The investigation was performed using both RF and GBM (i.e. XGBoost), whereas the findings are presented using feature importance. Each model will be evaluated providing the metrics introduced in the paragraph 3.4.2 of Materials and Methods section which are summarized in Table 13. The computer code has been implemented with Python programming language.

The results shown below are a partial view of the analysis, focused on motivating the methodological process followed during this study, a complete report of the analysis is available in the Supplementary Figures of Baldo and coll. paper (2023).

Model	Regression		Classification	
	R^2	MSE	R^2	Accuracy
RF + FS	$0.54 \pm 8.19 \cdot 10^{-5}$	154.31 ± 72.93		
RF + FS + DA	0.69 ± 0.00011	0.13 ± 0.047	$0.86 \pm 4.3 \cdot 10^{-5}$	0.66 ± 0.070
RF + FS + DA + AEM	0.70 ± 0.00019	0.12 ± 0.043	$0.86 \pm 0.17 \cdot 10^{-5}$	0.67 ± 0.076
XGB + FS + DA + AEM	$0.93 \pm 5.83 \cdot 10^{-5}$	0.11 ± 0.043	$0.96 \pm 8.61 \cdot 10^{-5}$	0.67 ± 0.094

Table 13. Evaluation of the models. *R2: coefficient of determination; MSE: mean squared error; RF: random forest; FS: feature selection; DA: data augmentation; AEM: age effect mitigation; XGB: XGBoost. Baldo et al. 2023*

4.4.1 Random forest combined with feature selection

Due to the scarcity of data samples and the high number of variables, we deployed a feature selection method aimed at reducing the number of random correlations in the dataset and removing the unimportant variables with reference to the predictive target (as described in paragraph 3.4.2 in Materials and Methods section and shown in Figure 2).

The application of the feature selection algorithm reduced the set of variables to a few candidates, where the predictor with higher importance is the chronological age of the subject. However, as reported in Table 13, the metrics reveal a bad performance of the model.

4.4.2 Random forest combined with feature selection and data augmentation

To counteract the absence of data points, we resorted to a form of data augmentation, as described in paragraph 3.4.3 of Materials and Methods section and shown in Figure 2. We are basically changing the feature space to obtain more samples and produce more accurate models.

This approach allows us to formulate the original learning problem in two forms: a regression task and a classification task. In the regression task, the predictive target is a continuous variable representing the AE score of the child. The predictor with higher importance is APGAR score at 1 min after birth, a measure of the physical condition of a newborn infant. The classification model has a discrete outcome, namely, a discrete numerical value obtained by the AE comparison in a pair of subjects, as described in paragraph 3.4.2 of Materials and Methods section. The categorical variables (e.g. hearing loss and duodenal atresia) gain higher importance.

Table 13 shows better performance metrics for the model (RF combined with feature selection and data augmentation) compared to the previous one (RF combined with feature selection). Even though we now have more reliable results, the quality of the information we retrieved through the analysis is debatable since the chronological age of the subject is highly related to the target of the model (AE) and might distort the real importance of other features. Moreover, this argument can be made for each variable that is known to be correlated to the chronological age of the children (e.g., creatinine level, homocysteine level, development milestones, height, occipital frontal circumference). To avoid this issue, we can mitigate the effect of age on the predictive model by applying the age effect mitigation method as described in paragraph 3.4.4 of Material and Methods section and shown in Figure 2.

4.4.3 Random forest combined with feature selection, data augmentation and age effect mitigation

In order to obtain an analysis not influenced by the chronological age of the subject, we resorted to the normalization method presented in paragraph 3.4.4 of Material and Methods section. The regression task (Fig. 13A) confirms the importance of the APGAR score at 1 min after birth and of almost all the other features compared to the previous model without age effect mitigation. The classification task (Fig. 13B) highlights another categorical variable related to gastrointestinal alteration (Hirschsprung disease). Both classification and regression tasks

show the importance of hearing loss, language (the month at which the subject started babbling), magnesium, immune system (CD19+ and immunoglobulins M) and vitamin B12. The evaluation score shows no significant variations demonstrating the robustness of the model (Table 13).

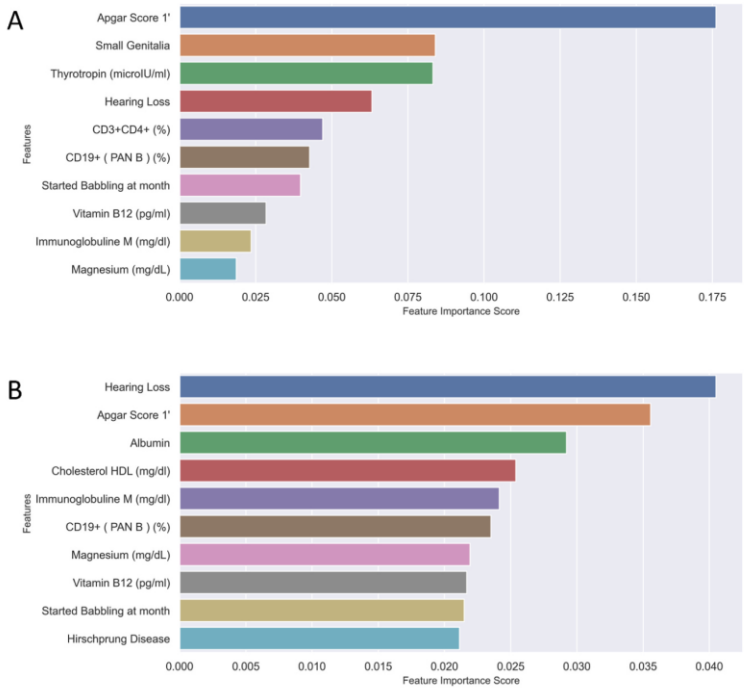

Figure 13. Random Forest combined with Feature Selection, Data augmentation and Age Effect Mitigation. *Feature importance obtained by random forest combined with feature selection, data augmentation and age effect mitigation. 3A. Regression task. 3B. Classification task. Baldo et al. 2023*

4.1.1 Gradient boosting machine combined with feature selection, data augmentation and age effect mitigation

XGBoost combined with Feature Selection and regression and classification tasks of XGBoost combined with Feature Selection and Data Augmentation model results are shown in Supplementary Figs 4, 5 and 6, respectively in Baldo et al. work (2023).

The XGBoost combined with all data manipulation steps confirms most of the results obtained by RF method. As shown in Fig. 14 A (regression task) the importance of language (the month at which the subject started babbling and hearing loss), small genitalia, thyrotropin, immune system (CD3+CD4+ and CD19+), and APGAR score at 1 min after birth are confirmed with a

higher R2 (R2 = 0.93, Table 2). Fig. 14B (classification task) shows again categorical variables related to gastrointestinal alterations (duodenal atresia, Hirschsprung disease).

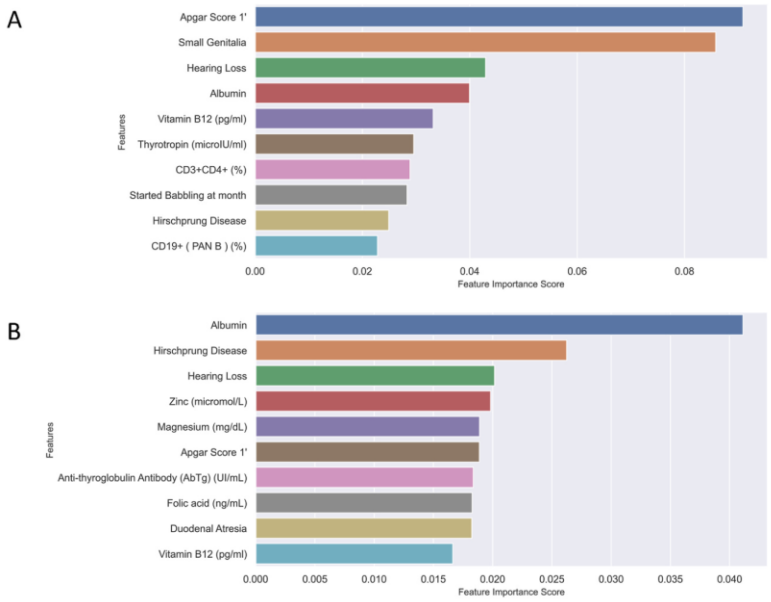

Figure 14. Gradient boosting machine combined with Feature Selection, Data augmentation and Age Effect Mitigation. *Feature importance obtained by gradient boosting machine combined with feature selection, data augmentation and age effect mitigation. 4A. Regression task. 4B. Classification task. Baldo et al. 2023*

4.2 One-carbon pathway metabolites dosage in children's plasma

The studied cohort included two groups:

- DS group consists of 164 subjects, 95 males (M) and 69 females (F), with a mean age of 11.55 years old (standard deviation, SD = 6.69) and an age range from 3.1 to 37.9; control group consists of 30 M and 24 F with a mean age of 14.53 years old (standard deviation, SD = 6.64) and an age range from 3.3 to 31.5; in this cohort, only one child has T21 mosaicism at 94%.
- Control group, 29 subjects were siblings of 23 subjects in the DS group.

In the DS group we obtained the concentration values of THF in 108 subjects, of 5-methyl-THF in 140 subjects, of 5-formyl-THF in 80 subjects, of SAH in 94 subjects and of SAM in 24 subjects. In the control group we obtained the concentration values of THF in 41 subjects, of 5-methyl-THF in 34 subjects, of 5-formyl-THF in 21 subjects, of SAH in 20 subjects and of SAM in 15 subjects (see Supplementary Data Sheet 1 of Vione et al. 2022 work). It was not possible to obtain the same number of measurements of metabolite levels in all the selected subjects for several reasons: the amount of plasma was not sufficient to perform ELISA assay for each metabolite, the absorbance (O.D., optical density) mean values of plasma samples were out of the range, or there was a technical problem during the assay. Moreover, it was not possible to have a larger group of SAM concentration results due to technical problems with some of the ELISA kits purchased.

Concerning the O.D. mean values, those that were higher or lower than the O.D. mean values of standard sample ranges were not taken into consideration (they are indicated in red in Supplementary Tables 1, 3–5 of Vione et al. 2022 work). In total we did not consider the concentration values of 9 DS plasma samples in THF ELISA assays, 55 DS and 8 control plasma samples in 5-formyl-THF assays, 18 DS and 14 control plasma samples in SAH ELISA assays because their detection rate was lower than the minimum of the standard range and 1 plasma sample in SAM ELISA assay because its concentration was higher than the maximum of the standard range.

In order to look for correlation between level of metabolites analysed by ELISA tests and levels of other available metabolites, for the DS group we collected the concentration values in serum of folic acid in 143 subjects and of vitamin B12 in 155 subjects and of plasma Hcy in 42 subjects.

Overall, 3 strong outliers were identified among 5-formyl- THF and vitamin B12 concentration values of the DS group and SAH concentration values of control group that are reported in red in Supplementary Data Sheet 1 of Vione et al. work (2022). The main results of descriptive analyses of THF, 5-methyl-THF, 5-formyl-THF, SAH and SAM without strong outliers are shown in Table 14.

	DS (*n* = 164)					Control (*n* = 54)				
	THF (ng/mL)	5-methyl-THF (ng/mL)	5-formyl-THF (pg/mL)	SAH (ng/mL)	SAM (ng/mL)	THF (ng/mL)	5-methyl-THF (ng/mL)	5-formyl-THF (pg/mL)	SAH (ng/mL)	SAM (ng/mL)
Valid *n* =	108	140	79	94	24	41	34	21	19	15
Missing n =	56	24	85	70	140	13	20	33	35	39
Mean	41.574	50.247	151.675	6.588	10.308	57.783	45.369	145.917	1.911	8.022
Median	33.955	49.766	137.955	6.298	9.514	51.471	42.744	139.144	1.736	5.222
SD	29.414	9.358	29.300	2.923	4.237	32.645	12.301	15.413	1.213	6.362

Table 14. Main results of descriptive analysis of DS and control group excluding strong outliers*. The values of subjects with Down syndrome (DS) are reported on the left of the table and the values of normal control subjects (control) are reported on the right of the table. For each metabolite the number (n) of plasma samples valid or missing are reported. Also the mean, median and standard deviation (SD) values are shown. The detailed results of descriptive analysis with and without strong outliers are shown in Supplementary Table 6 of Vione et al. work (2022). Vione et al. 2022.*

The results of unpaired student t-test between DS and control concentration values of each metabolite showed that the difference between DS and control concentrations is statistically significant for THF (p-value = 0.0041), 5-methyl-THF (p-value = 0.015) and SAH (p-value = 0.0001) when the strong outliers are excluded (see Table 15) or included (see Supplementary Table 7 of Vione et al. work (2022)) in the analyses. The results of Mann-Whitney test between DS and control 5-formyl-THF concentration values is not statistically significant when the strong outliers are included (p-value = 0.88076) or excluded (p-value = 0.81034).

Figure 15 shows the variation of THF, 5-methyl-THF, 5- formyl-THF, SAH, and SAM concentrations in subjects with DS and normal control subjects excluding strong outliers. In Supplementary Figure 1 of Vione et al. work (2022) the variation of 5-formyl-THF and SAH concentration in subjects with DS and normal control subjects is reported including strong outliers.

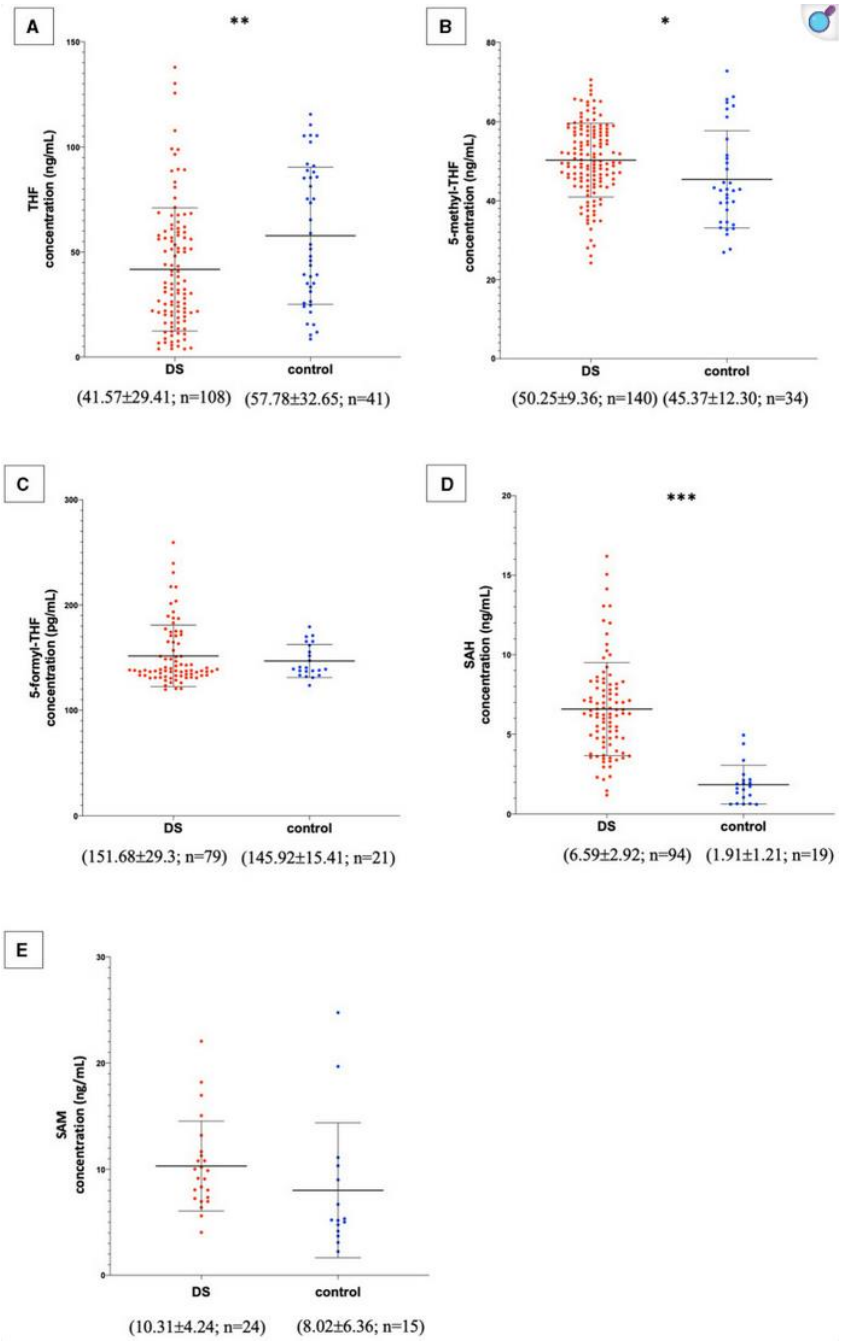

Figure 15. Metabolite concentrations in subjects with DS and normal control subjects. *The graphs report metabolite plasma levels of each subject in the study. On the x-axis there is the subdivision of subjects in DS and control groups. Subjects with DS are represented like red dots and normal control subjects are represented like blue dots. On the y-axis the concentration of the metabolite in ng/mL or pg/mL is reported. The asterisks above the graph indicate the level of statistical significance (∗p < 0.05; ∗∗p < 0.005; ∗∗∗p < 0.0005). The middle black lines indicate the mean concentration values for each group and the external black lines indicate standard deviation (SD) values. The mean concentration, SD values and the number of subjects (n) are reported below each graph for DS and control groups. (A) shows THF concentrations; (B) shows 5-methyl-THF concentrations; (C) shows 5-formyl-THF concentrations excluding strong outliers; (D) shows SAH concentrations excluding*

strong outliers; (E) shows SAM concentrations. The graphs were created with GraphPad Prism software v.6.0 (San Diego, CA). Vione et al. 2022

In Table 15 the ratio of mean concentration values and the ratio of median concentration values between DS and control groups is reported. A statistically significant weak correlation (r = Pearson correlation coefficient) was found between age and THF concentration levels in control group (r = 0.395 and p-value = 0.011). The significance was lost in DS group.

	THF	5-methyl-THF	5-formyl-THF	SAH	SAM
Subjects	DS (n = 108)	DS (n = 140)	DS (n = 79)	DS (n = 94)	DS (n = 24)
	Control (n = 41)	Control (n = 34)	Control (n = 21)	Control (n = 19)	Control (n = 15)
Mean ratio DS/Control	0.72	1.11	1.04	3.45	1.28
Median ratio DS/Control	0.66	1.16	0.99	3.63	1.82
p-value	0.0041*	0.0115*	0.8103	0.0001*	0.1853
t-value	2.9137	2.5537	801[a]	6.8373	1.3499

Strong outliers are excluded. Mean and median concentration values are given in Table 1. Ratio of mean and median values between DS and control groups are given. Statistical test was t-student except for 5-formyl-THF for which Mann-Whitney test was used. Significant p-values (<0.05) are marked with an "*". The detailed results with and without strong outliers are shown in Supplementary Table 7.
[a]U-value for Mann-Whitney test.

Table 15. Difference of metabolite concentration between DS and control groups. *Vione et al. 2022*

Whether or not the strong outliers were considered did not change the results (see, respectively Table 16 and Supplementary Table 8B of Vione et al. work (2022)). A statistically significant moderate correlation was found between age and Hcy concentration levels in the DS group (r = 0.593 and p-value < 0.001) (see Supplementary Tables 8A,B of Vione et al. work (2022)).

	DS					Control				
	THF	5-methyl-THF	5-formyl-THF	SAH	SAM	THF	5-methyl-THF	5-formyl-THF	SAH	SAM
r	0.089	-0.085	0.128	0.152	0.099	0.395	0.271	0.239	0.102	0.115
p-value	0.362	0.318	0.261	0.145	0.644	0.011*	0.121	0.296	0.678	0.682

The table reports for each metabolite the results of the statistical analyses in DS and control groups indicated by Pearson correlation coefficient (r) and two-tailed significance (p-value). Significant r (>0.4) and p-values (<0.05) are marked with an "*". The detailed results of bivariate correlation with and without strong outliers are shown in Supplementary Table 8.

Table 16. Bivariate correlation between age and each concentration level in DS and control groups excluding strong outliers. *Vione et al. 2022*

The unpaired t-test did not identify differences between males and females concerning the concentration of all the molecules investigated (see Supplementary Table 9 of Vione et al. work (2022). The unpaired t-tests comparing values in fasting or non- fasting state highlighted a statistically significant difference in vitamin B12 levels in the DS group when the strong outliers are both included (p-value = 0.004) (see Supplementary Table 10A of Vione et al. work (2022)) or excluded (p-value = 0.005) (see Supplementary Table 10B of Vione et al. work (2022)) in the analyses.

The linear correlation analysis identified a statistically significant moderate negative correlation ($r = -0.628$ and p-value = 0.029) between SAM and vitamin B12 levels in the non-fasting DS group. A statistically significant strong positive correlation ($r = 0.81$ and p-value = 0.003) between SAM and 5-formyl-THF levels was found in DS group. The correlation was not maintained in the control group (see Supplementary Table 11 of Vione et al. work (2022), Figure 16 and Supplementary Figure 2 of Vione et al. work (2022)).

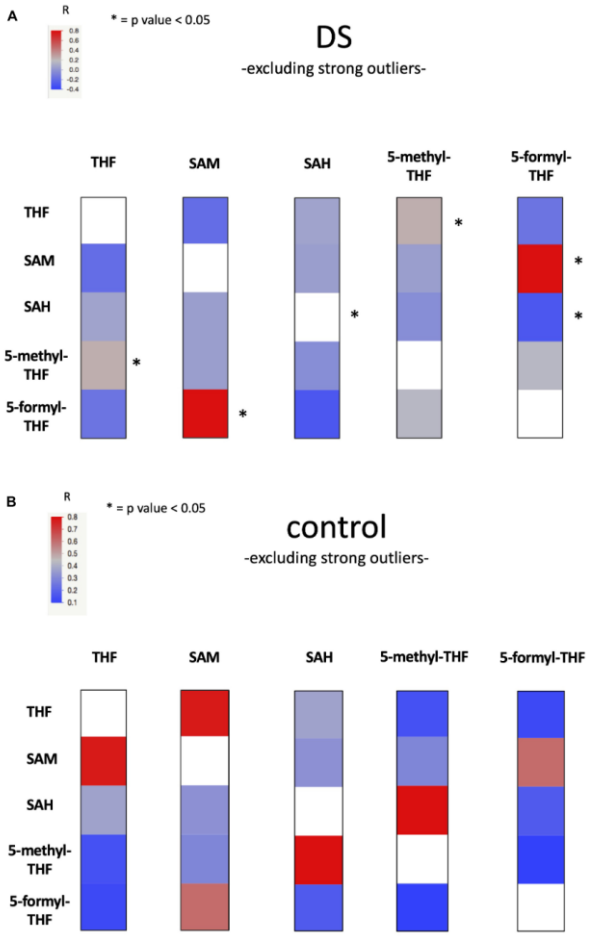

Figure 16. ***Heat Map figure of bivariate correlation between levels of each metabolite and levels of all the other metabolites excluding strong outliers.*** *(A) presents bivariate correlations in DS group and (B) presents bivariate correlation in the control group (for complete data see Supplementary Table 11). At the top left of the figures the color code bar for Pearson correlation coefficient (r). Statistically significant correlations were marked with an "∗". Vione et al. 2022*

4.3 Analysis of metabolic and genetic imbalance of the homocysteine-methionine cycle in trisomy 21

4.3.1 Case selection

The studied cohort included a total of 106 subjects were selected for this study, including 58 subjects with DS and 48 subjects as control group (N), selected among siblings of subjects with DS and without evidence of abnormal karyotype.

For this work, we considered in the DS group subjects with a diagnosis of DS with homogeneous or mosaic T21, the availability of an adequate amount of urine to determine SAM and SAH metabolites, and we recorded whether the samples were collected in a fasting or not-fasting state. The urine samples were collected from subjects with a similar mean age as close as possible to the N group. Concerning the N group, we considered subjects who do not have pathologies, availability of an adequate amount of urine to determine SAM and SAH metabolites, and similar mean age as close as possible to the DS group. The urine samples selected were treated within two hours of collection and with an apparent good physical state (clear urine after centrifugation).

DS group consists of 35 males (M) and 23 females (F) with a mean age of 12.72 years old (yrs) (standard deviation, SD=5.92) and age range from 3 to 28 yrs; the N group consists of 32 M and 16 F with a mean age of 14.42 yrs (standard deviation, SD=7.10) and an age range from 2 to 31 yrs. Concerning the N group, 20 subjects were siblings of 17 subjects in the DS group. For every collected sample, parents filled out a form with information about the current fasting state, last meal, concomitant diseases and consumed medications (Supplementary dataset 1).

4.3.2 Comparison between plasma and urine levels of SAM and SAH and statistical analyses

Considering that children's hydration status can affect the metabolites analyzed in urine samples, uSAM and uSAH concentrations (µM) were adjusted for specific gravity (SG).

In the DS group, uSAM median concentration value was 8.33 µM (range: 4.89 – 13.15), uSAH median concentration value was 0.58 µM (range: 0.24 – 1.07) (Table 17 and 18, Supplementary dataset 1). DS uSAM/uSAH ratio was 14.36. In the N group, uSAM concentration median value was 8.41 µM (range: 4.73 – 14.70), uSAH concentration median value was 0.48 µM (range: 0.22 – 0.88) (Table 17 and 18, Supplementary dataset 1). The N uSAM/uSAH ratio was 17.52.

	DS				N			
	SAH concentration (µM) in urine adjusted for specific gravity	SAM concentration (µM) in urine adjusted for specific gravity	SAH concentration (nM) in plasma Vione et al. 2022	SAM concentration (nM) in plasma Vione et al. 2022	SAH concentration (µM) in urine adjusted for specific gravity	SAM concentration (µM) in urine adjusted for specific gravity	SAH concentration (nM) in plasma Vione et al. 2022	SAM concentration (nM) in plasma Vione et al. 2022
Mean	0.59	8.87	16.96	21.64	0.51	8.81	5.40	14.95
SD	0.18	2.21	8.08	4.55	0.19	2.39	4.83	7.28
Median	0.58	8.33	16.56	21.53	0.48	8.41	4.19	13.01

Table 17. S-adenosyl-methionine (SAM) and S-adenosyl-homocysteine (SAH) concentration values. Mean, median and standard deviation (SD) are showed for each metabolites in plasma and urine of Down syndrome (DS) and control (N) groups.

	Urine		Plasma	
	SAH	SAM	SAH	SAM
Mean ratio DS/N	1.16	1.01	3.14	1.45
Median ratio DS/N	1.21	0.99	3.95	1.66
p-value	0.021	0.913	< 0.001	0.019

Table 18. ***S-adenosyl-methionine (SAM) and S-adenosyl-homocysteine (SAH) concentration ratios.*** *The ratios between DS and N mean and median values of SAM and SAH are showed. The results of unpaired t-test for SAH and SAM concentration values in plasma and in urine samples both in DS and N groups are also showed.*

The levels of the urine metabolites analyzed follow a normal distribution and are not affected by fasting state, sex or age with the exception of uSAH levels, which have a moderate statistically significant correlation with age in both DS ($p=0.002$ and $R=0.405$) and N groups ($p=0.004$ and $R=0.413$). Regarding the same analyses performed in plasma data recently published (selected for the subjects of which we have urine quantifications), metabolite levels follow a normal distribution, and are not affected by fasting state, sex or age (Supplementary table 1A, 1B, 1C, 1D).

We have found that the concentration of uSAH is statistically slightly higher in the DS group vs N ($p=0.021$, Cohen's $D=0.19$) with a DS/N median ratio of 1.21; concerning the concentration of uSAM, we did not see a difference between DS and N (median ratio=0.99, $p=0.913$, Cohen's $D=2.258$). An increase of SAH and SAM levels was also observed in plasma samples of subjects of which we have urine quantifications, in particular a DS/N median ratio of 3.95 ($p<0.001$, Cohen's $D=7.221$) for pSAH and of 1.66 ($p=0.019$, Cohen's $D=6.169$) for pSAM (Table 17 and 18, Supplementary table 1E).

Analyzing the correlation of metabolite levels (with age as covariate) in urine, a moderate/strong correlation between uSAH and uSAM is present both in DS (r=0.593; p<0,001) and in N (r=0.628; p<0,001).

The descriptive analyses performed with SPSS statistics on plasma and urine metabolites did not highlight the presence of strong outliers among the analyzed values.

4.4 One-carbon pathway metabolites involvement in cognitive development in children with DS

Analysing the distribution of THF, 5-f-THF, 5-m-THF and SAH none of them showed a normal distribution and no strong outliers were found.

No difference in metabolites levels emerged between sexes.

4.4.1 Correlations between metabolites and cognitive scores

As shown in Table 19 partial correlation checked for age were performed between cognitive scores of Griffiths and WPPSI test and metabolites level. A moderate statistically significant correlation was found between 5-m-THF and subscale D (personal-social-emotive scale) of Griffiths test.

		Griffiths-III test					WPPSI IV test		
		Foundation of learning	Language	Fine-Motor	Socio-Emotional	Gross-Motor	Verbal Domain	NonVerbal Domain	Total Score
5-f-THF (pg/mL)	Pearson's r	-0.179	-0.148	-0.156	-0.354	-0.111	-0.303	-0.174	-0.296
	p value	0.559	0.630	0.629	0.259	0.745	0.124	0.387	0.134
	n	14	14	13	13	12	28	28	28
5-m-THF (ng/mL)	Pearson's r	0.447	0.398	0.256	0.576	0.162	-0.318	0.113	-0.206
	p value	0.072	0.113	0.322	0.016	0.581	0.081	0.554	0.267
	n	18	18	18	18	15	32	32	32
THF (ng/mL)	Pearson's r	-0.086	-0.100	0.010	0.075	0.380	0.162	0.163	0.237
	p value	0.744	0.703	0.972	0.783	0.147	0.391	0.390	0.207
	n	18	18	17	17	17	31	31	31

SAH (ng/mL)	**Pearson's r**	0.005	-0.114	0.300	0.021	0.301	0.096	0.386	0.213
	p value	0.987	0.725	0.344	0.949	0.341	0.687	0.093	0.367
	n	13	13	13	13	13	21	21	21

Table 19. Partial correlation between metabolites level and cognitive score. *Statistically significant correlation are highlighted in red. Age equivalent score for Griffiths and WPPSI are reported. 5-f-THF=5-formil-tetrahydrofolate; 5-m-THF=5-methyl-tetrahydrofolate, THF=tetrahydrofolate; SAH=S-adenosyl-homocysteine; A AE G= Foundations of learning; B AE G=Language and Communication B; C AE G= Fine-Motor Skills ; D AE G=Adaptove-Socio-emotional Skills; E AE G= Gross-Motor skills; n= number of subjects*

Using contingency tables we analysed association between metabolites level and IQ scores as shown in Table 20. Metabolites levels were considered as high or low compared to 25°, 50° and 75° percentiles threshold and IQ score was considered as high or low compared to the median of the analysed group. The statistically significance was obtained with Fisher's exact test. A high or low level referred to the 25° and 50° percentile of 5-f-THF is associated with IQ high of low scores referred to the median. Other association were found between high or low level referred to the 50° percentile of 5-m-THF and IQ high of low scores referred to the median and high or low level referred to the 25° percentile of THF and IQ high of low scores referred to the median.

Variable 1	**Variable 2**	**n**	**Fisher's test p value bilateral**
5-f-THF 25° H/L	**IQ G H/L**	14	0.031
5-f-THF 50° H/L	**IQ G H/L**	14	0.031
5-f-THF 75° H/L	**IQ G H/L**	14	1.000
5-f-THF 25° H/L	**IQ W H/L**	28	0.069
5-f-THF 50° H/L	**IQ W H/L**	28	0.276
5-f-THF 75° H/L	**IQ W H/L**	28	1.000
5-m-THF 25° H/L	**IQ G H/L**	18	0.137
5-m-THF 50° H/L	**IQ G H/L**	18	0.049
5-m-THF 75° H/L	**IQ G H/L**	18	0.367

5-m-THF 25° H/L	**IQ W H/L**	32	0.472
5-m-THF 50° H/L	**IQ W H/L**	32	1.000
5-m-THF 75° H/L	**IQ W H/L**	32	0.338
THF 25° H/L	**IQ G H/L**	18	0.013
THF 50° H/L	**IQ G H/L**	18	0.050
THF 75° H/L	**IQ G H/L**	18	0.245
THF 25° H/L	**IQ W H/L**	31	1.000
THF 50° H/L	**IQ W H/L**	31	0.285
THF 75° H/L	**IQ W H/L**	31	1.000
SAH 25° H/L	**IQ G H/L**	13	1.000
SAH 50° H/L	**IQ G H/L**	13	0.217
SAH 75° H/L	**IQ G H/L**	13	0.608
SAH 25° H/L	**IQ W H/L**	21	0.056
SAH 50° H/L	**IQ W H/L**	21	0.400
SAH 75° H/L	**IQ W H/L**	21	0.606

Table 20. Fisher's exact test metabolites level considered as high or low compared to 25°, 50° and 75° percentiles threshold and IQ level considered as high or low compared to the median. *Statistically significant correlation are highlighted in red. n= number of subjects; H/L= high or low, IQ=intellectual quotient; G=Griffiths test assed to children between 3-6.99 years old, W=WPPSI test assessed to children between 7-16 years old*

4.4.2 Correlations between metabolites and executive functions and adaptive behaviour scores

We analysed partial correlations checked by age between one-carbon metabolites and Adaptive behaviour scores (Table 21) and Executive function scores (Table 22).

Considering adaptive behavior no statistically significant correlations emerged with studied metabolites both in preschooler children group and in school aged children group. Focusing on

		Preschooler children					School-aged children			
		Comm.	Daily Living	Social	Motor	Composite IQ	Comm.	Daily Living	Social	Composite IQ
5-fTHF (pg/mL)	Person's r	-0,067	-0.351	-0.130	0.042	-0.164	-0.327	-0.129	-0.274	-0.265
	p value	0.828	0.240	0.671	0.891	0.592	0.096	0.520	0.166	0.181
	n	14	14	14	14	14	28	28	28	28
5-mTHF (ng/mL)	Person's r	0.406	0.221	0.470	-0.119	0.148	-0.084	0.019	0.079	0.024
	p value	0.106	0.393	0.057	0.651	0.571	0.652	0.917	0.673	0.896
	n	18	18	18	18	18	32	32	32	32
THF (ng/mL)	Person's r	0.007	-0.046	-0.029	0	-0.120	0.092	-0.137	0.306	0.089
	p value	0.978	0.860	0.911	0.999	0.646	0.628	0.470	0.100	0.641
	n	18	18	18	18	18	31	31	31	31
SAH (ng/mL)	Person's r	0.156	0.108	0.386	0.298	0.247	0.251	0.169	0.380	0.250
	p value	0.629	0.738	0.215	0.348	0.439	0.285	0.478	0.098	0.287
	n	13	13	13	13	13	21	21	21	21

Table 21. Partial correlations between metabolites level and Vineland scores. *Statistically significant correlation are highlighted in red. Pre-school children =children aged 3 to 6.99 years old, School-aged children= children between 7 and 16.99 years old, n=number of subjects, V=Vineland, G=Griffiths test, IQ=Intellectual Quotient, W=WPPSI. Comm=Communication skills, Daily=Daily Living skills, Social=Social-emotive skills. Motor=Motor skills*

		BRIEF-P					BRIEF-2								
		Inhibition	Shift	Emotion	WM	Plan	Inhibition	Self Monitor	Shift	Emotion	Initiate	WM	Plan	Task	O.M.
5-fTHF (pg/mL)	Person's r	-0.023	-0.206	0.190	-0.130	-0.029	0.175	0.279	0.211	0.351	0.423	0.336	0.082	0.206	0.170
	p value	0.944	0.520	0.555	0.687	0.929	0.383	0.159	0.29	0.073	0.028	0.087	0.683	0.303	0.397
	n	13	13	13	13	13	28	28	28	28	28	28	28	28	28
5-mTHF (ng/mL)	Person's r	-0.038	-0.439	-0.086	0.090	-0.158	-0.072	-0.022	-0.007	-0.194	0.182	0.077	0.112	-0.099	0.208
	p value	0.887	0.089	0.752	0.742	0.559	0.705	0.908	0.969	0.304	0.336	0.687	0.556	0.601	0.269
	n	17	17	17	17	17	31	31	31	31	31	31	31	31	31
THF (ng/mL)	Person's r	0.105	-0.259	0.069	0.135	-0.122	-0.427	-0.384	-0.004	-0.452	-0.158	-0.402	-0.138	-0.383	-0.035
	p value	0.698	0.332	0.798	0.617	0.652	0.021	0.040	0.983	0.014	0.414	0.031	0.474	0.040	0.856
	n	17	17	17	17	17	30	30	30	30	30	30	30	30	30
SAH (ng/mL)	Person's r	0.540	0.352	0.175	0.602	0.403	-0.089	-0.386	-0.028	-0.280	0.223	-0.166	0.325	-0.136	0.266
	p value	0.086	0.288	0.607	0.050	0.220	0.717	0.103	0.910	0.246	0.360	0.498	0.175	0.579	0.272
	n	12	12	12	12	12	20	20	20	20	20	20	20	20	20

Table 22. Partial correlations between metabolites level and Executive functions scores. *Statistically significant correlation are highlighted in red. BRIEF-P and BRIEF-2 are reported as T scores. n=number of subjects, Emotional= Emotional regulation, WM= Working memory, Plan= Plan/Organization, Task= Task Monitoring O.M.= Organization of Material; N=number of subject*

executive functions, in preschooler children no significant correlation emerged while, on the other hand, analyzing the correlation of metabolite levels and executive function in school aged children, a moderate statistically significant correlation emerged between THF level and Inhibition (r=-0.427 p=0.021), Emotion Regulation (r=-0.452 p=0.014) and Working Memory (r=-0.402 p=0.031) and between 5-f-THF and Initiate scale (r=-0.423, p=0.028). A mild-moderate correlation was found between THF level and Self Monitor scale (r=-0.384 p=0.040) and THF level and Task Monitoring scale (r=-0.383 p=0.040).

5. Discussion

5.1 Understanding cognitive profile and development trajectories of children with Down syndrome

In order to be able to study the influence of metabolic processes on cognitive development, we decided as a first step to analyse the cognitive data in our possession to further our knowledge of the cognitive profile in children with trisomy 21.

From our studies, a marked interindividual variability emerged within our sample as a whole, revealing three sub-groups of much the same size with different cognitive profiles:

1) One group of 21 participants had similar verbal and non-verbal skills (the Homogeneous Profile group) and obtained higher global cognitive scores than the other two subgroups.
2) A second group of 22 participants (the Non-verbal Profile group) had lower scores in the verbal than in the non-verbal domain and had the features of the profile mostly described in the literature, with a better performance in non-verbal processing than in the verbal domain. This group of participants showed the lowest cognitive level and reached developmental milestones later in life. We might surmise that this was the group in which genes had the most impact on the individuals' cognitive profile.
3) The third group of 29 children (the Verbal Profile group) obtained better results in the verbal domain (with scores as high as in the Homogeneous Profile group) than in the non-verbal one (their scores being similar to those of the Non-Verbal Profile group).

These results explain the marked interindividual variability identified, both within groups and between different studies, suggesting that it would be better to assume that individuals with DS can express not just one, but multiple different cognitive profiles.

Moreover, our findings tend to be in line with the work of Tsao and Kindelberg published in 2009. They identified four profiles in childhood, three of which correspond to those emerging in our study with their group with similar scores for verbal and non-verbal processing coincides with our Homogeneous Profile group, and showing one exception: whereas the scores obtained in the subtests of the two indices were much the same in our group, they saw worse score in Classification subtest, which is part of the Non-Verbal Index, than in the other. Their second group scored better in non-verbal subtests, like our Non-Verbal Profile group. Their third group obtained significantly higher scores in verbal subtests, like our Verbal Profile group. In our

study we did not find any cluster corresponding to their fourth group, which featured verbal scores close to the mean, and lower than non-verbal scores. This difference might be due to the tasks used to assess verbal and non-verbal skills, as some were similar (e.g., their "Vocabulary" task corresponded to our "Picture Naming" task), while others differed (e.g., none of the tasks we administered resembled their "Social comprehension" task). Another possible explanation would concern environmental variables that might have shaped participants' cognitive profiles differently.

As indicated by Schalock in 2010, difficulties with adaptive behaviour define the grade of intellectual disability and, in our sample all children with DS had standardized scores at least two standard deviations below the norm in one or more adaptive behaviour domains, with most showing deficits in multiple areas. This aligns with previous findings (e.g., Will et al. 2018) and is consistent with the intellectual disability associated with DS. When comparing the two age groups, preschoolers had higher standardized scores than school-aged children with DS in Daily Living Skills and Socialization. The lower scores in older children reflect not a loss of abilities, but rather a slower development of these skills compared to typically developing peers. Communication scores were equally low in both age groups, consistent with the well-documented language development challenges individuals with DS face throughout their lives (Silverman, 2007; Grieco et al. 2015).

Focused on Executive Functions across different life stages, our data aligns with the literature. For preschool children, Emotional Control is typically a strength, while Working Memory is a notable weakness (Loveall et al. 2017). Skills like Shift, Plan/Organise, and Inhibit fall between these two extremes. In school-aged children, Emotional Control and Organisation of Materials emerge as the strongest abilities, with Inhibit and Self-Monitor showing intermediate levels. The weakest areas include Working Memory, Monitor, Plan/Organise, and Shift (Daunhauer, Fidler, Hahn et al. 2014; Lee et al. 2011, 2015; Loveall et al. 2017). Differences between the two age groups were observed in Plan/Organise and Shift, where older children exhibited greater weaknesses. This may be due to the increasing difficulty of tasks in these areas as children age, or because school-related demands make these challenges more noticeable to parents, whereas they may have previously gone undetected (Loveall et al. 2017). The two age groups differed in the areas of Plan/Organise and Shift, where older children demonstrated more pronounced weaknesses. This could be attributed to the increasing difficulty of tasks in these domains as children age, or to heightened school demands that make these challenges more noticeable to parents, whereas they may have gone unnoticed earlier (Loveall et al. 2017). This supports the hypothesis that environmental factors, rather than age alone, may influence the Executive Function profile of children with Down syndrome.

The relationship between Executive Functions (EFs) and adaptive behaviour varied across the two age groups in this study. For preschoolers, the two domains appeared largely independent, with significant connections only between Communication and Working Memory, the latter predicting the former. In contrast, for school-age children, correlations were found between almost all EFs and adaptive behaviour domains, highlighting the critical role of EFs in daily functioning at this stage. The Emergent Metacognition Index predicted Communication, with Working Memory as the key predictor, likely due to the established link between working memory and language, seen in both typical development (Gathercole, 2006) and DS (Lanfranchi et al. 2009). The Behaviour Regulation Index, particularly Inhibit, predicted Daily Living Skills, likely because the ability to inhibit impulsive responses is essential for tasks like self-care, household chores, money management, time management, and following rules. Additionally, Inhibit and Shift were predictors of Socialization, as inhibition is crucial for monitoring one's behaviour in social interactions, and shifting is important for adapting to changing social contexts and applying appropriate social rules.

Studying the developmental trajectories, from our data children with DS continue to acquire new AF skills, depending on the AF domain, as they grow up, although the gap between them and their peers tends to widen, too. Our analyses confirmed the trend found by Will et al. 2018 for Communication, Daily Living Skills, and Socialization domains. In particular, for communication the scores tended to increase linearly with age, for daily living skills after 75 months, and for socialization after 80 months of CA or 39 months of MA, the speed of skill acquisition decreased might be due to the increasing demands that the environment poses to children who need more time to acquire and consolidate new skills. Moreover, our data highlighted that individual characteristic such as cognitive level, as measured in terms of MA, and experience, as measured by CA (assuming that older children have more experience of life than younger ones), have an impact in supporting the development of AF skills. AF seems to develop beyond MA, and while the trajectory of MA tends to flatten at 128 months, AF tends to increase and this suggest that cognitive functioning supports the development of AF skills, but the environment influences and promotes their development (Onnivello et al. 2024).

5.2 A Machine Learning approach to study influence of clinical and metabolic data on intellectual disability

The increasing availability of big data and of electronic health records has paved the way for Artificial Intelligence (AI) in the medical field, in particular with the using of Machine learning (ML) approaches.

ML models can help researchers identify key features likely associated with ID in DS and may ultimately enhance efforts to discover potential therapeutic targets. ML approaches are particularly useful for addressing two challenges linked to complex conditions like DS. First, they can handle the integration of diverse and mixed data types. Second, ML methods can uncover non-linear relationships between various data types, allowing for hypothesis-free modelling to reveal new pathways involved in cognitive impairment in DS. Nikam and coll. in 2019 used a ML algorithm to identified disease-specific and phenotype-specific thresholds of analytes, such as low tyrosine/large neutral amino acids, high citrulline/arginine ratios etc, for differential diagnosis of mild, moderate, and severe intellectual disabilities (Nikam et al. 2019). Another model was built by Donnelly and colleagues (2023) to identify a compact set of psychiatric and physical health measures that differentiate individuals with a neurodevelopmental disorder from controls and highlight higher-order structure within these measures.

The results of our analyses show that ML algorithms can be applied with good accuracy to identify variables likely involved in cognitive delay in DS. In this context it is very important to take into account the effect of chronological age on the outcome. Considering all the steps described in methods section (tree-based models and Boruta framework, feature importance tasks, age effect mitigation, data enrichment) we have shown two well performing models (RF and XGBoost both combined with feature selection, data augmentation and age effect mitigation) with low error (MSE <0.12) and an acceptable R2 (0.70 and 0.93) (Table 13). XGBoost is providing the best performance for both the regression (R2 = 0.93, MSE = 0.11) and the classification task (R2 = 0.96, Accuracy = 0.67).

Interestingly, the variables of importance show several features that can be considered with particular attention during the follow up of DS patients. In particular, in the regression tasks, the results show two groups of variables of importance (Figs. 13A and 14A). First, different characteristics might be grouped in perinatal and neonatal status and development. The APGAR score at 1 min after birth is a measure of the physical condition of a newborn infant that can influence the neonatal development (Del Hoyo Soriano et al. 2021). Hearing loss has been demonstrated to affect language and cognitive skills development (Laws and Hall 2014). This might slow down the achievement of developmental milestones, such as babbling (Fig. 13A, B and 14A), that could be predictors of later motor, cognitive skills and language (Locatelli et al. 2021). The ML models are consistent with the idea that early developmental milestones can be important variables to consider in planning early phenotype-informed interventions that have the potential to influence positive trajectories in community living and participation across an individual's lifespan. Finally, thyrotropin concentration can be related to thyroid disorders that can affect the nervous system and play a role in cognitive delay (Khaleghzadeh-Ahangar et al. 2021).

The second group of variables of importance is related to the immune system (e.g. CD19+ and immunoglobulins M) underlining the documented immune dysfunction typical of DS (Verstegen et al. 2010). In the classification tasks (Fig. 14A–B), among the top ten variables of importance we find variables related to gastrointestinal alterations (e.g. Hirschsprung disease and duodenal atresia). Variables related to gastrointestinal disorders can be related to microbiome alterations. Since there are increasing evidence that the composition of the resident bacteria within the gastrointestinal tract can influence cognitive functions (Gareau 2016), treatments for gastrointestinal disorders and modulation of the microbiota should be deeper investigated. Moreover, the presence of congenital gastrointestinal alterations can lead to a surgical operation in the first stages of life, and this may influence child's development in his newborn life. Lastly, results obtained through both RF and XGB models show the importance of vitamin B12: a concentration threshold of vitamin B12 has been highlighted to be important in the DS population which might have greater vitamin requirements (Antonaros et al. 2021). It is known that cognitive delay may be exacerbated by the presence of chronic health conditions (Gandy et al. 2020).

Individuals with DS and co-occurring chronic disorders may benefit from early interventions to mitigate their risk for adverse cognitive outcomes: for this reason, it is very important to identify important variables that may affect cognitive development and to use these data to improve care pathways, in particular perinatal care.

5.3 One-carbon pathway metabolites and cognitive development in children with DS

The analyses we performed highlighted plasma level alteration of some intermediates of one-carbon metabolism in a group of subjects with DS compared to a group of euploid subjects as control. We reported a statistically significant difference of THF (p-value = 0.0041), 5-methyl-THF (p-value = 0.015) and SAH (p-value = 0.0001) plasma levels between DS and control groups (see Table 15). Concerning 5-formyl-THF and SAM, the difference is not statistically significant (respectively p-value = 0.8103 and 0.1853) (see Table 15).

THF plasma concentration is lower than normal in subjects with DS and the median concentration ratio between DS and control groups is 0.66 (see Table 15), that is a 2:3 ratio, strongly suggesting a correlation with the imbalanced original event or the presence of a third Hsa21. Moreover, THF concentration level shows a statistically significant weak correlation with age in the control group (r = 0.395 and p-value = 0.011) (see Table 16). Pfeiffer and coll.

reported that older age is associated with less bioactive folate (THF) and more biologically inactive folate (Pfeiffer et al. 2015). Interestingly, our results report that the correlation between THF and age is lost in the DS group ($r = 0.089$ and p-value = 0.362) suggesting that the plasma level alteration of THF in subjects may be a stable consequence of trisomy 21 masking variation with age.

Considering 5-methyl-THF and 5-formyl-THF plasma concentration they showed a median concentration ratio between DS and control groups respectively of 1.16 and 0.99 (see Table 15), that is a 1:1 ratio.

SAM and SAH were analysed both in plasma in Vione et al. work (2022) together with the former one-carbon pathway metabolites and in urine with a second work (Vione et al. 2024, submitted).

SAH plasma concentration is much higher than normal in subjects with DS and the median concentration ratio between DS and control groups is 3.63 (see Table 15). Even if there is not a statistically significant difference of SAM plasma levels between the DS and control groups due to the distribution of the values (see Table 15), SAM concentration is higher than normal in subjects with DS and the median concentration ratio between DS and control groups is 1.82 (see Table 15). We find here that, following data in Table 14, the SAM/SAH mass median ratio is 1.511 in subjects with DS and 3.008 in control subjects, suggesting that SAM/SAH median ratio in subjects with DS is half (exactly 0.50) compared to control subjects. Our statistical analyses report a negative correlation between SAM concentration levels and vitamin B12 (when the subjects are in a non-fasting state) ($r = -0.628$ and p-value = 0.029) and a positive correlation between SAM and 5-formyl-THF ($r = 0.81$ and p-value = 0.003) in plasma samples in the DS group (see Figure 16 and Supplementary Table 11 of Vione et al. 2022). These findings are difficult to interpret but it is interesting to note that the association between SAM and 5-formyl-THF is present only in the DS group and is lost in the control group, suggesting again a selective alteration of one-carbon cycle in subjects with DS. Further studies are necessary to increase the number of SAM concentration values obtained from DS and control groups.

Analysing SAM and SAH in urine (uSAM and uSAH), we found that the median concentration of uSAH is slightly higher in the DS group with a DS/N median ratio of 1.21, while no difference was found in uSAM between DS and N. As previously found in plasma analysis, a decrease of the SAM/SAH ratio was found also in urine. Concerning urine, the SAM/SAH ratio is 80% lower in DS than in the N (Table 17).

The comparison between the uSAH and uSAM levels and in a different biological fluid as plasma (pSAH, pSAM) in DS and N groups showed very interesting differences. uSAH levels

are slightly increased in DS subjects (DS/N median ratio= 1.21, p-value= 0.021) (Table 17B), while uSAM levels are similar between the two groups. pSAH and pSAM levels are also higher in DS subjects (DS/N median ratio= 3.95 and 1.66 respectively, p-value< 0.001 and 0.019) (Table 17B). These data show that pSAH DS/N median ratio vs. uSAH DS/N median ratio is 3.26 times higher, so there is a greater discrepancy of the pSAH levels between DS and N groups than the uSAH levels allowing us to speculate an excess of pSAH which is not excreted by urine. A similar pattern exists for SAM, indeed pSAM DS/N median ratio vs. uSAM DS/N median ratio is 1.68 times higher. Bivariate correlation analyses between uSAH levels and uSAM levels both in DS and N groups show that the increase of SAH is linked to the increase of SAM ($r=0.593$, $p<0.001$ for DS group; $r=0.628$; $p<0,001$ for N group) (Supplementary table 1F).

The major excess of SAH in DS vs. N than SAM in both urine and plasma fluids might be considered a metabolic alteration typical of DS. The excess of both molecules in DS might be responsible of some phenotypic aspects because of their documented pro-apoptotic effect (Zhang and Zheng 2021).

SAM/SAH ratio is a well-known indicator of cellular methylation capacity and when it is decreased compared with a control condition can correlate with alteration of the methylation potential (Clarke 2001, Petrossian and Clarke 2011). We detected that the SAM/SAH ratio in the plasma of subjects with DS is half of that in the N group (0.5) confirming the alteration of the methylation capacity reported in DS.

Another interesting information is the lack of the relationship between SAH or SAM and homocysteine levels in plasma samples. These data indicate that the relationship between the increase of both SAH and homocysteine in plasma of euploid subjects reported by Clarke (2021) does not occur in DS.

Analysing the influence of one-carbon metabolites on cognitive scores we found that 5-f-THF, 5-m-THF and THF seem to be associated in different ways to high or low intelligent quotient scores (see Table 20). In particular, in older children, THF level seems to be correlated with different aspects of executive functioning like inhibition, working memory, self and task monitoring and emotion regulation (see Table 22) hypothesizing that an increasing of THF level could improve executive functioning scores. Considering the absence of statistically significant correlations in preschooler children, this means that an alteration of THF level could show his effects over the years and could lead to a higher difficulty in executive functioning whether they are seen as strengths (e.g. emotion regulation) or weaknesses (e.g. working memory). Considering the other metabolites, 5-m-THF seems to be correlated with Social and Emotion domain of cognitive profile while 5-f-THF has a moderate correlation with Initiate

skills. At the moment we could not hypothesize the meaning of these correlations but further analyses could help understand the underlying link.

5.4 Limits and future perspectives

This study provides valuable insights into the cognitive profile of children and adolescents with DS and its association with one-carbon pathway, but it has some limitations.

First, like many studies on neurogenetic syndromes, the sample size is relatively small, limiting the statistical analysis and the potential use of machine learning techniques. Enrolling more subjects and including more centres could help increasing the cohort of patient giving the possibility of increasing the statistical power of the analyses.

Second, the sample was recruited from a single clinical centre, which, while ensuring data consistency, may reduce the diversity of the sample. Including multiple centres could introduce greater heterogeneity, accounting for variables like therapy quality and home environment, and enable external validation of the identified clusters.

At least, the study uses cross-sectional data rather than a longitudinal approach, which limits the ability to explore changes in EFs, adaptive behaviour, and their interaction over time. Future research should adopt longitudinal methods to provide a clearer picture of developmental trajectories.

6. Conclusions

In conclusion we were able to deepen our knowledge regarding the cognitive profile of children with trisomy 21. In particular, we were able to highlight the presence of 3 different cognitive profiles and confirmed, among the executive functions, which represent strengths and weaknesses. We have seen, moreover, how executive functions could influence the development of adaptive skills and that while children with DS continue to acquire adaptive skills over time, the gap with typically developing peers widens, influenced by both cognitive level and environmental factors.

As we have seen, the use of new technologies can be useful in the study of complex and very multifaceted aspects such as cognitive, and the machine learning algorithm we have developed may be able to help in both research and clinical support by highlighting important cognitive developmental features to be considered.

Moreover, our results confirm that there is a dysregulation of the one-carbon pathway in subjects with DS that could be related to cognitive impairment. In particular, it is remarkable that plasma THF median concentration in subjects with DS appears to be lacking and exactly inversely proportional to the Hsa21 chromosomal dosage, implying a direct role of Hsa21 in impairing the progression of the folate/one-carbon cycle. In addition, considering the correlation present between the levels of the above metabolite and the executive functions of children with trisomy 21 we can hypothesize that it plays an important role in the cognitive development of such individuals. These founding open the possibility that restoring a normal THF concentration could be important in subjects with DS. To this aim, we could evaluate administration of THF itself, or of the well-known folinic acid (5-f-THF), or of 5-methyl-THF.

Studies have shown that THF, like folic acid, does not effectively counteract MTX toxicity in both euploid and T21 fibroblast cells. In contrast, treatments with 5-methyl-THF and 5-formyl-THF have demonstrated significantly better protective effects during MTX exposure (Vitale et al., 2019). Additionally, the THF branch of the one-carbon metabolic network is distant from the key steps related to methylation capacity, which is underscored by the altered SAM/SAH ratio previously observed in DS and confirmed in this study. Lastly, it's important to note that THF supplements are currently not available for human use.

Folinic acid has been used the longest for treating CFDs (Irons et al., 1986) and remains the most commonly used form of folate for this purpose (Pope et al., 2019). However, studies have clearly shown that it does not improve cognitive skills in children with DS (Blehaut et al., 2010). This outcome aligns with the role of folinic acid in the folate cycle, where it tends to

convert to more oxidized forms of THF, whereas the Hcy/methionine cycle requires more reduced forms.

5-methyl-THF is the most reduced form of folate (Nelson et al. 2017), the biologically active form, and is crucial for the production of SAM, the universal methyl donor, by regenerating methionine from Hcy (see Figure 1). SAM acts as a potent methylating agent in various biosynthetic reactions, with its methyl group being the most reactive in the one-carbon cycle (Nelson et al. 2017). Notably, 5-methyl-THF is the only form of folate capable of crossing the blood-brain barrier (Ducker and Rabinowitz, 2017). It is well absorbed in the intestines, with its bioavailability unaffected by additional enzymatic steps (Pope, 2019). Although available for human use since 2000 (Venn et al., 2002), it remains underutilized. One reason for this is its higher cost compared to folic acid, despite potential advantages. It may be superior to folic acid, especially in bypassing the conversion pathway to 5-methyl-THF, which is beneficial when the methylation of THF is impaired, such as in individuals carrying the MTHFR C6777 polymorphism (Greenberg and Bell, 2011).

The use of 5-methyl-THF has more recently been proposed for the treatment of CFD (Li et al. 2008, Knowles et al. 2016) and is considered to be the most efficient way to normalize CSF 5-methyl-THF concentrations (Pope 2019). Although 5-methyl-THF plasma concentration is not decreased in the subjects with DS that we have investigated, from the above discussion it appears to be the best way to restore the THF deficit. In addition, considering hat in subjects with CFD the peripheral blood 5-methyl-THF levels may be normal while they are low in the CSF (Pope 2019), a similar condition might be present in DS. Even if a draw of CSF only for research appears unjustified to us, we plan to study 5-methyl-THF levels in samples available following neurosurgery in subjects with DS.

For these reasons, we recommend 5-methyl-THF as the most promising candidate for a clinical trial aimed at improving cognitive function in individuals with DS. However, we caution against its use until a pilot study determines the effective dosage needed to correct the altered values.

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