Expanding the phenotype of Kleefstra syndrome: speech, language, and cognition in 103 individuals

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ABSTRACT

Objectives Speech and language impairments are core features of the neurodevelopmental genetic condition Kleefstra syndrome. Communication has not been systematically examined to guide intervention recommendations. We define the speech, language, and cognitive phenotypic spectrum in a large cohort of individuals with Kleefstra syndrome.

Method 103 individuals with Kleefstra syndrome (40 males, median age 9.5 years, range 1-43 years) with pathogenic variants (52 9q34.3 deletions, 50 intragenic variants, 1 balanced translocation) were included. Speech, language, and non-verbal communication were assessed. Cognitive, health and neurodevelopmental data were obtained.

Results The cognitive spectrum ranged from average (12/79, 15%) to severe (12/79, 15%). Language ability also ranged from average (10/90, 11%) to severe (53/90, 59%). Speech disorders occurred in 48/49 (98%) verbal individuals and even occurred alongside average language and cognition. Developmental regression occurred in 11/80 (14%) individuals across motor, language, and psychosocial domains. Communication aids, such as sign and speech generating devices, were crucial for 61/103 (59%) individuals including those who were minimally verbal, had a speech disorder, or following regression.

Conclusions The speech, language, and cognitive profile of Kleefstra syndrome is broad, ranging from severe impairment to average ability. Genotype and age do not explain the phenotypic variability. Early access to communication aids may improve communication and quality of life.

Keywords: Kleefstra Syndrome, Chromatin, Phenotype, Language, Speech, Intellectual Disability, Regression, Autism Spectrum Disorder

What is already known on this topic

Kleefstra syndrome is a rare, neurodevelopmental condition caused by loss-of-function of *EHMT1*, a chromatin remodelling gene. Communication disorders are reportedly common in Kleefstra syndrome, yet these have been poorly characterised to date.

What this study adds

We provide systematic characterisation of speech, language and cognitive abilities in a large cohort of individuals with Kleefstra syndrome. We reveal that speech and language disorders are a core feature of Kleefstra syndrome, even in the absence of intellectual disability.

How this study might affect research, practice or policy

Our delineation of specific speech and language disorders in this cohort paves the way for the first targeted behavioural speech therapies in this group. Our work underscores the critical need for early speech and language intervention including use of communication aids, and the need for lifelong access to tailored therapy supports.

INTRODUCTION

Kleefstra syndrome (OMIM 610253) is a rare, neurodevelopmental condition caused by loss-of-function of *EHMT1* (euchromatic histone lysine methyltransferase 1, HGNC: 24650) a chromatin remodelling gene. It typically arises due to *de novo* intragenic variants and 9q34.3 deletions, but can be inherited from a mildly affected or mosaic parent (1). Large deletions (>1Mb) are associated with a more severe phenotype than smaller deletions (<1Mb) and intragenic *EHMT1* variants (2).

Kleefstra syndrome is a multisystemic condition with neurological, cardiac, musculoskeletal, gastrointestinal, renal, and urogenital features. While most individuals have intellectual disability, there are two cases reported with average cognition (3, 4). Autism spectrum disorder (autism), speech and language disorders, epilepsy, sleep disturbance and mental health disorders are common (2, 5). Communication skills have been captured using subjective clinical descriptors (using symbols, understanding simple sentences) (2, 6), rather than systematic evaluations with standardised tools.

Given speech and language impairment is a core aspect of Kleefstra syndrome, characterization of these communication deficits is critical for diagnostic and prognostic counselling and informs therapeutic approaches. We provide the first comprehensive analysis of speech, language, adaptive behaviour, and cognition in a large cohort (*N*=103) of individuals with Kleefstra syndrome.

PATIENTS AND METHODS

Participants

Participants were included if aged over 6 months and had 9q34.3 deletions or pathogenic or likely pathogenic intragenic variants affecting *EHMT1.* Participants or their families self-referred to the study through Kleefstra syndrome support organisations (see Acknowledgements) or were referred by their treating clinician in response to a study flyer distributed throughout international clinical genetic networks. Parents or legal guardians provided written informed consent for minors or adult participants with cognitive impairment. Adult participants of testamentary capacity provided written informed consent. Study materials were available in English, Dutch, German, French, Italian, Spanish, and Portuguese. Ethics approval was obtained from the Royal Children's Hospital Human Research Ethics Committee (HREC 37353A).

Health, Development and Feeding

Participants completed a health and development questionnaire, used in other genetic studies (7-12). English-speaking primary caregivers of children younger than 7 years completed the Child Oral Motor Proficiency Scale (ChOMPS) (13). Caregivers of participants with sialorrhea completed the Drooling Impact Scale (14). Cognitive data was obtained, where available, from assessments completed by participants' local clinicians felt to be more valid for this population than online cognitive testing, this also reduced participant burden.

Adaptive Behaviour

The standardised Vineland Adaptive Behaviour Scales 2nd (Vineland II; French) and 3rd Edition (Vineland III; English, Spanish) assessed adaptive behaviour, language, daily living, socialisation and motor skills (15, 16). Normative data for motor skills ceases at 6 years 11 months (Vineland II) and 9 years 11 months (Vineland III). As no participants reached the ceiling on the tool, motor skills of older participants were assessed using the oldest available normative data. The Adaptive Behaviour Composite score (ABC) provided an overall score of the language, daily living, and socialisation domains.

Regression

Caregivers completed the Development and Neurobehavioral Regression (DANR) questionnaire to identify presence, length and triggers of regression across language, social and motor skills (17).

Language and social communication

Participants who verbally communicated their daily needs and used sentences were considered ‘verbal’. Participants with few, single or no spoken words were described as ‘minimally verbal’. English and Dutch-speaking caregivers of verbal participants older than 4 years completed the Children’s Communication Checklist 2nd Edition (CCC-2) (18). The CCC-2 is norm referenced and measures communication skills across 10 subdomains (Supplementary Figure 1). No participants reached the ceiling on the CCC-2 so 16-year-old normative data was used for older participants. As

aforementioned, the Vineland II/III assessed receptive, expressive, and written language skills.

English and Dutch-speaking caregivers of children older than 2 years completed the Social Responsiveness Scale 2nd Edition (SRS-2) (19). The SRS-2 assesses social behaviour (social awareness, cognition, communication, and motivation) and restricted and repetitive behaviours, based on autism diagnostic criteria (20). A higher total T-score indicates more autistic behaviours.

Augmentative and alternative communication

Caregivers described augmentative and alternative communication (AAC, also known as communication aids) modalities, and their perception of AAC utility. AAC was defined as unaided (not using an external system) or aided (using an external system). Minimally verbal participants' communication was assessed using the Communication Matrix (21). The Communication Matrix assesses communication behaviours seen in typical development prior to 24-months of age across four communication functions and seven communication levels (Supplementary Figure 2).

Speech

A speech pathologist used a comprehensive battery to differentially diagnose speech disorders in English-speaking, verbal individuals via videoconference. This battery included single word stimulus (repeating words three times to assess consistency), a conversation sample and a brief oral motor exam (22). Speech disorders were operationalised as phonological delay (speech sound errors seen in typical

development in >10% of younger children), phonological disorder (speech sound errors not observed in typical development occurring in <10% of younger children), articulation disorder (distorted speech sound production, e.g., a lisp), childhood apraxia of speech (CAS), dysarthria, and stuttering. CAS is a disorder of motor planning and programming diagnosed using the American Speech, Language and Hearing Association's three core diagnostic criteria: i) inconsistent production of consonants and vowels, ii) lengthened and disrupted coarticulatory transitions, and iii) inappropriate prosody (23, 24). Dysarthria is a neuromuscular disorder of speech production, characterised by describing features of speech subsystems; respiration, phonation, resonance and articulation (25). Lastly, presence and severity of stuttering were characterised using stuttering severity ratings (0=no stuttering to 9=severe stuttering) (26). To obtain an overall rating of how well an individual is understood by different communication partners (e.g., teachers, family, strangers), participants completed the Intelligibility in Context Scale (ICS) which provides a Likert scale from 1 (never understood) to 5 (always understood) (27).

English, Dutch, French, German, Italian, Portuguese and Spanish-speaking verbal participants also completed speech tasks via the Redenlab® digital speech platform, which were acoustically analysed (tasks and methodology available in Supplementary Table 1) (28).

Statistical analysis

Mann-Whitney U-tests were used to examine differences between two independent samples (e.g., Vineland ABC scores between individuals with deletion genotypes and intragenic variants), and Wilcoxon Signed-rank tests were used for two

dependent samples (e.g., receptive and expressive language skills). For group comparisons, such as ABC scores across four different intragenic variant groups (nonsense, frameshift, splice site, missense), a Kruskal-Wallis test was used. Linear regression assessed relationships between two continuous quantitative variables (e.g., standardised scores and age).

RESULTS

Participants

The cohort included 103 participants (40 male, 39%) from 26 countries, who were studied at a median age of 9.5 years (range: 19 months – 43.5 years) (Table 1, Figure 1, Supplementary Table 2 for genotypic and demographic details). The median age of diagnosis was 3 years (Q1-Q3 1 year 1 month – 8 years). Fifty-two participants had 9q34.3 deletions, 50 participants had intragenic variants, and 1 participant had a heterozygous balanced translocation interrupting *EHMT1*. Of the participants with 9q34.3 deletions, 7 were >1Mb, 34 were <1Mb, and for 11 participants the size of their deletion was not available. Intragenic variants included 17 frameshift, 16 nonsense, 8 splice site and 9 missense variants. Missense variants located within the ANK-repeat (*n*=6), pre-SET (*n*=1) and SET domains (*n*=2). Seventy-five cases were confirmed *de novo* and inheritance for 28 cases was unknown. One participant inherited their variant due to paternal mosaicism (Participant 96). Participants 74 and 75 were sisters. Participant 33's deletion was associated with a ring chromosome 9 and participant 42's deletion was mosaic (>70%). Participant 20 had 47, XYY. Participant 64 also had a paternally inherited

missense variant in *JAG1* and had a dual diagnosis of Kleefstra and Alagille syndrome.

Table 1. Genotypes and phenotypes of 103 individuals with Kleefstra syndrome

	CHROMOSOMAL DELETIONS			INTRAGENIC VARIANTS				TOTAL COHORT [#]
	>1Mb	Other[†]	<1Mb	Nonsense	Frameshift	Splice site	Missense	
N[‡]	7	11	34	17	16	8	9	103
Male	3/7, 43%	5/11, 46%	15/34, 44%	6/17, 35%	4/16, 25%	4/8, 50%	3/9, 33%	40/103, 39%
Age at assessment (median)	9 years	16 years	10 years	10 years	9 years	7 years	6 years	9 years
Age of genetic diagnosis (median)	1 years	5 years	2 years	3 years	3 years	7 years	2 years	3 years
NEURODEVELOPMENTAL CONDITIONS								
Autism	2/7, 29%	3/11, 27%	12/34, 35%	10/17, 59%	5/16, 31.3%	3/8, 38%	4/9, 44%	39/103, 28%
Attention deficit hyperactive disorder	1/7, 14%	1/11, 9%	6/34, 18%	2/17, 12%	4/16, 25%	1/8, 13%	1/9, 11%	16/103, 16%
Sensory processing disorder	3/7, 43%	2/11, 18%	7/34, 21%	4/17, 33%	4/16, 25%	1/8, 13%	3/9, 33%	24/103, 23%
Specialist childcare/kinder	3/7, 43%	1/11, 9%	4/34, 12%	3/17, 18%	0/14, 0%	1/8, 13%	1/9, 11%	13/101, 13%
Mainstream childcare/kinder	0/7, 0%	1/11, 9%	6/34, 18%	4/17, 24%	5/14, 36%	1/8, 13%	4/9, 44%	21/101, 21%
Specialist primary school	1/7, 14%	2/11, 18%	8/34, 24%	2/17, 12%	3/14, 21%	2/8, 25%	1/9, 11%	20/101, 20%
Mainstream primary school	1/7, 14%	2/11, 18%	4/34, 12%	2/17, 12%	1/14, 7%	2/8, 25%	1/9, 11%	13/101, 13%
Specialist high school	2/7, 29%	3/11, 27%	7/34, 21%	4/17, 24%	3/14, 21%	0/8, 0%	1/9, 11%	20/101, 20%
Mainstream high school	0/7, 0%	1/11, 9%	2/34, 6%	2/17, 12%	1/14, 7%	2/8, 25%	1/9, 11%	9/101, 9%
Home school	0/7, 0%	1/11, 9%	2/34, 6%	0/17, 0%	1/14, 7%	0/8, 0%	0/9, 0%	4/101, 4%
Tertiary education[¶]	0/7, 0%	0/11, 0%	1/34, 3%	1/17, 6%	1/14, 7%	1/8, 13%	1/9, 11%	5/101, 5%
REGRESSION OCCURED[§]	1/6, 17%	1/6, 17%	2/24, 8%	4/15, 27%	0/14, 0%	2/8, 25%	1/7, 14%	11/80, 14%
EPILEPSY	2/7, 29%	2/11, 18%	5/34, 15%	2/17, 12%	1/16, 6%	0/8, 0%	0/9, 0%	12/103, 12%
SLEEP DISTURBANCE	6/7, 86%	4/11, 36%	22/34, 65%	8/17, 47%	13/16, 81%	3/8, 38%	8/9, 89%	65/103, 63%
Frequent waking	6/7, 86%	3/11, 27%	16/34, 47%	7/17, 41%	10/16, 63%	3/8, 38%	7/9, 78%	53/103, 51%
Early waking	2/7, 29%	1/11, 9%	8/34, 24%	2/17, 12%	3/16, 19%	1/8, 13%	1/9, 11%	19/103, 18%

Difficulty falling asleep	2/7, 29%	2/11, 18%	5/34, 15%	1/17, 6%	1/16, 6%	1/8, 13%	1/9, 11%	13/103, 13%
Extended sleepless periods	1/7, 14%	2/11, 18%	2/34, 6%	0/17, 0%	0/16, 0%	0/8, 0%	0/9, 0%	5/103, 5%
Sleep apnoea	0/7, 0%	0/11, 0%	0/34, 0%	0/17, 0%	2/16, 13%	0/8, 0%	0/9, 0%	2/103, 2%
CARDIAC PROBLEMS	4/7, 57%	5/11, 46%	11/34, 32%	3/17, 8%	6/16, 38%	2/8, 25%	2/9, 22%	33/103, 32%
VISION IMPAIRMENT	6/7, 86%	6/11, 55%	17/34, 50%	11/17, 65%	10/16, 63%	6/8, 75%	6/9, 67%	62/103, 60%
HEARING IMPAIRMENT	6/7, 86%	2/9, 22%	10/34, 29%	9/16, 56%	6/16, 38%	1/8, 13%	3/9, 33%	37/100, 37%
Mild	1/7, 14%	1/9, 11%	7/34, 21%	3/16, 19%	2/16, 13%	1/8, 13%	1/9, 11%	16/100, 16%
Moderate	4/7, 57%	1/9, 11%	3/34, 9%	4/16, 25%	4/16, 25%	0/8, 0%	1/9, 11%	17/100, 17%
Severe	1/7, 14%	0/9, 0%	0/34, 0%	0/16, 0%	0/16, 0%	0/8, 0%	1/9, 11%	2/100, 2%
Profound	0/7, 0%	0/9, 0%	0/34, 0%	1/16, 6%	0/16, 0%	0/8, 0%	0/9, 0%	1/100, 1%
Conductive	1/7, 14%	1/9, 11%	2/34, 6%	1/16, 6%	4/16, 25%	0/8, 0%	2/9, 22%	11/100, 11%
Sensorineural	2/7, 29%	1/9, 11%	2/34, 6%	3/16, 19%	1/16, 6%	1/8, 13%	0/9, 0%	10/100, 10%
Mixed	3/7, 43%	0/9, 0%	6/34, 18%	3/16, 19%	1/16, 6%	0/8, 0%	1/9, 11%	14/100, 14%
Unknown type	0/7, 0%	0/9, 0%	0/34, 0%	1/16, 6%	0/16, 0%	0/8, 0%	0/9, 0%	1/100, 1%
Unknown severity and type	0/7, 0%	0/9, 0%	0/34, 0%	1/16, 6%	0/16, 0%	0/8, 0%	0/9, 0%	1/100, 1%

† = other deletions detected by fluorescence in situ hybridisation, or without specific location and size of deletion specified in chromosomal microarray report, ‡ = denominators reflect how many individuals have provided data/were assessed for each area, § = assessed with the Developmental and Neurobehavioral Regression (DANR) questionnaire, ¶ = college diploma, trade apprenticeship or university degree. # = 1 individual with heterozygous balanced translocation disruption *EHMT1* also included in total cohort number.

Denominators reflect how many individuals have provided data/were assessed for each area. See Supplementary Tables 2, 3 and 4 for further genotypic, demographic, and phenotypic details.

Health, Development and Feeding

Health conditions

The health and medical profile of the cohort was broad and differed for some features amongst genotypes (see Table 1, Supplementary Table 3, Supplementary Results 1 for further details). Complications during pregnancy (42/103, 41%) and birth (59/103, 57%) were common.

Approximately one-third of participants had cardiac conditions (33/103, 32%). Many participants were affected by constipation (41/103, 40%) and reflux (12/103, 12%). More than half of participants had dental conditions (59/103, 57%). Six participants had hypothyroidism.

Half the cohort had a history of ear infections (52/103, 50%), and many had persistent hearing impairment (37/100, 37%). Sixteen participants had hearing aids but many (7/16, 44%) did not tolerate wearing them. Participants 40 and 97 had progressive hearing loss. Vision impairment was relatively common (62/103, 60%).

Feeding

Infant feeding impairment occurred in 35/103 (34%) individuals. Infants were breast (69/103, 67%) and/or bottle-fed (53/103, 51%). Supplemental nasogastric feeding was required in 10/103 (10%) and/or gastrostomy tube feeding in 5/103 (5%) individuals. Many participants (75/103, 73%) had feeding impairment in early childhood (<8 years), persistent in several participants (23/58, 40%) older than 8 years. Of 61/103 (59%) with drooling ranging from mild to severe on the Drooling Impact Scale, 34/61 had ongoing drooling while it had resolved in 27/61. Total eating

and drinking skills on the ChOMPS (*n*=29) were below the 5th centile for age in 20/29 (69%) individuals. Complex movement patterns (e.g., using the tongue to lick corners of the mouth) of 19/29 participants were of high concern (<5th centile). Most participants had average basic movement patterns (e.g., holding a bottle, 27/29), fundamental oral skills (e.g., closing lips completely, 25/29), and oral motor coordination (e.g., moving jaw up and down to chew, 22/29).

Neuropsychiatric features

Sleep disturbance occurred in 65/103 (63%) of participants (Table 1). Epilepsy occurred in 12/103 (12%) of participants. Heterogeneous nonspecific findings on MRI brain studies were found in 38/79 (48%) individuals (Supplementary Table 3).

Mental health symptoms were common. Although four participants had a formal diagnosis of anxiety disorder (4%), 24/103 (23%) of the cohort were reported as being anxious. Fewer participants had depressive symptoms (7/103, 7%) while 2/103 (2%) had obsessive-compulsive disorder, and one had (1%) bipolar disorder (Supplementary Table 3). Obsessive behaviours occurred in 25/103 participants (24%) and 10/103 (10%) had phobias. Behavioural concerns were common (43/103, 42%,) and included aggression (19/103, 18%), self-harm (18/103, 17%), impulsivity (18/103, 17%), hyperactivity (11/103, 11%) and attention problems (27/103, 26%).

Of the 79/103 (77%) who had a cognitive assessment, 67/79 (85%) had intellectual disability (Figure 2, Supplementary Table 3): Mild (16/79, 20%), moderate (38/79, 48%), severe (12/79, 15%) (for one individual, the severity was not specified). Average cognitive ability was observed in 12/79 (15%) individuals (confirmed by report: 45, 50, 67, 70, 72, 73, 76, 83, 85, 100, self-reported/reported

by caregiver: 48, 74), including 5 with borderline intellect. Participant 48 had completed post-graduate studies at University. Participant 74 had an ABC score of 93 (ABC average range >85), attended a mainstream school and was at expected level for all subjects. Other diagnoses included autism, sensory processing disorder and attention deficit hyperactive disorder (ADHD), and social communication disorder (Table 1, Supplementary Table 3). Developmental coordination disorder occurred in 14/103 (14%) individuals, and 3/103 (3%) had cerebral palsy (Supplementary Table 3).

Milestones

Motor milestones were protracted (Supplementary Table 4). All participants could sit apart from participant 1 (>1Mb deletion) who was not yet sitting at 4 years. 62/102 participants (61%) crawled at ≥14 months and 90/102 walked at ≥16 months (88%). Seven participants aged 1-to-4-years-old were still learning to walk. All participants older than 2-years-old who were not yet walking had a deletion genotype.

Language milestones mirrored motor skill delays. 40/102 (39%) were older than 18 months when they said their first word. All participants with missense and frameshift genotypes had said their first words. 17/102 (17%) had not yet said their first words, aged 1-to-28-years-old, and 41/102 (40%) participants were not combining words. For those participants who combined words, this usually began at 4-5 years (35/102, 34%). Milestones were not known for one participant.

Therapy and education

Participants attending specialist and mainstream education settings are noted in Table 1. Five adult participants had completed tertiary education. Participants 73 and

90 were completing a degree and diploma at college, respectively. Participant 97 completed a diploma, and participant 67 completed a trade apprenticeship. Participant 48 had completed post-graduate qualifications from university. Participants attended occupational therapy (87/101, 86%), physiotherapy (90/101, 89%) and speech therapy (100/103, 97%). Eighteen caregivers and adults voluntarily identified an affinity for music.

Adaptive Behaviour

Eighty-eight participants completed the Vineland III and two completed the Vineland II (total *n*=90) (Figure 1a, Figure 1b, Supplementary Figure 3). Vineland II/III domain scores across all domains ranged from low (20-70) to average (>85) (normative mean=100, normative SD=15). Average daily living skills (mean=58.79, SD=19.92), socialisation (mean=61.41, SD=21.99), and motor skills (mean=66.78, SD=16.40) were low. There was not a significant difference between communication, socialisation, daily living skills or motor skill domains (p=0.09). The overall adaptive behaviour composite score (ABC, normative mean=100, normative SD=15) reflected domain scores (mean=60.77, SD=18.39). In total, five participants had an average ABC score (Participants 19, 59, 74, 90, 100). Of these five participants, two had average cognition, two had a mild intellectual disability and one individual had not been formally assessed for cognitive ability. For those with Vineland ABC scores 3 standard deviations below the mean (<55, *n*=30), 5 participants had a severe intellectual disability, 14 moderate, 3 mild, and 1 with an unknown level of intellectual disability. Six participants had not been formally assessed for cognition, and one participant had average cognitive ability.

Participants with >1Mb deletions had the lowest ABC scores (Supplemental Figure 3). ABC scores were not significantly different amongst >1Mb, <1Mb and unspecified deletion groups (p=0.32) or between nonsense, frameshift, splice site and missense variants (p=0.92). Participants with deletion genotypes (*n*=46) had lower ABC scores than participants with intragenic variants (*n*=43, p=0.004). Whilst ABC scores trended downward with age (Supplementary Figure 4), only a small proportion of ABC scores variance was explained by age (R^2=0.14).

Deletion location did not appear to impact the ABC scores of participants with deletions (Figure 1a). Similarly, location of nonsense, frameshift and missense variants did not appear to impact ABC scores (Figure 1b). The ABC scores of female and male participants also did not differ (p=0.41).

Regression

Eleven participants had experienced regression (11/80, 14%) according to the DANR questionnaire. Individuals lost skills between 1 to 28 years of age, across language (9/11) (Supplementary Results 1), social (7/11) and fine (4/11) and gross motor (4/11) skills. Participants regained some language (7/9), social (5/7), and fine (4/4) and gross motor (4/4) skills. Regression triggers reported by parents (8/11) included illness (3/11), seizures (3/11), and life changes (e.g., leaving school, moving house, 4/11). Three participants had multiple regression triggers and sleep was impacted for two participants during regression. Participants experienced regression onset in adolescence and early adulthood (between 12 to 28-years-old, 6/11), or at younger than 4-years-old (5/11). Regression duration ranged from 1 to 5 years.

Four participants (4/11) reported more than one period of regression, such as short periods of losing and then regaining skills.

Language skill loss in younger children was described as beginning to say sounds and words, and then ceasing to perform this skill. In adolescents and young adults, language regression included previously using an aided AAC system to create five words sentences, to not using the aided AAC system, or participants going from using spoken sentences (~10 words in length) to single words.

Language and Social Communication

Average language skills were low (mean=59.20, SD=2.90; normative mean=100, SD=15) as measured by the Vineland II/III communication domain (*n*=90, Figure 2, Supplementary Figure 3). Communication subdomains of receptive (mean=8.54, SD=4.70; normative mean=15, SD=3), expressive (mean=8.31, SD=4.49) and written language skills (mean=7.11, SD=3.94) were also low. Ten participants had communication domain scores in the average range (Participants 12, 19, 56, 59, 70, 74, 90, 93, 100, 101). Receptive and expressive language skills were not significantly different (p=0.46). Little of the variance in communication ability was due to age (R^2=0.059). There was no significant difference in language performance (communication domain scores) for individuals with sleep disturbance compared with those without sleep disturbance (p=0.12).

Speech was the most impaired of the CCC-2 subdomains (*n*=44, normative mean=10, SD=3, mean=2.75, SD=2.80, Supplementary Figure 1). Speech was significantly lower ($p<0.05$) than 7/9 subdomains. The next most impaired was syntax. All subdomain scores ranged from low to average.

Total SRS-2 T scores (*n*=73, <60 within normal limits, 60-65 mild, 66-75 moderate, >75 severe; Figure 2, Supplementary Figure 5) ranged from within normal limits (8/73, 11%), to mild (13/73, 18%), moderate (24/73, 33%), and severely impaired (28/73, 38%). Social awareness, social cognition, social communication, and restricted and repetitive interest subdomains were moderately impaired. Social motivation was mildly impaired and was significantly stronger than social awareness, cognition, and communication (p=0.005).

Augmentative and Alternative Communication

AAC use was common (61/103, 59%). Several participants used gestures and signs younger than 2-years-old (85/103, 83%). Aided AAC was used in early childhood (3 to 5 years, 41/97 42%; 6 to 10 years, 24/70, 34%), as was sign and gesture (3 to 5 years, 73/97, 75%; 6 to 10 years, 27/70, 39%). Children between 11 and 15 years also used sign and gesture (12/42, 29%), and aided AAC (8/42, 19%). Participants 16 years and older used aided AAC (8/28, 29%) and gesture and sign (9/28, 32%) at similar rates. Four participants began using AAC again after regression impacted speech and language skills.

Fifty-three participants (51%) had used sign with use ranging from single signs (33/103, 32%) to simple (14/103, 14%, 4-to-26-years-old), and more complex sentences (6/103, 6%, 5-to-36-years-old, Figure 3). Some participants who used simple (7/14, 50%) and complex sign sentences (2/6, 33%) had hearing loss. Participants with aided AAC (37/103, 36%, Figure 3) used low-tech (16/103, 16%), high-tech (17/103, 17%), or a combination of low- and high-tech systems (4/103, 4%). Participant 1 was learning an eye-gaze aided AAC system. All other participants

accessed their aided AAC systems directly (i.e., finger point). Of the participants who used sign or aided AAC, many could also speak in sentences (22/103, 21%). Twenty percent of caregivers (19/94, 20%) believed that AAC hinders speech development.

The Communication Matrix (*n*=41) identified strengths in refusing and obtaining. Across communication levels on average, participants could perform 71.76% of refusing communication skills, and 61.59% of obtaining communication skills. Communicating for social (on average participants could perform 42.89% of skills) or information functions (on average participants could perform 31.47% of skills) were relative weaknesses. On average, participants could communicate to refuse, obtain and for social reasons using concrete symbols (Supplementary Figure 2). Only two participants (Participants 53, 68) could combine signs or symbols (level 7 on the Communication Matrix) for refusing, obtaining, social and information functions.

Speech

Most verbal, assessed participants had a speech disorder (48/49, 98%, Figure 4). The motor speech disorders of dysarthria (34/49, 69%) and CAS (29/49, 59%) were common, and often co-occurred (16/49, 33%). Dysarthria features included monopitch (19/34, 56%) and monoloudness (19/34, 56%), breathy voice quality (19/34, 56%), and hyper- (16/34, 47%) and hypo-nasality (11/34, 32%). Articulation disorder (21/49, 43%), phonological delay (18/49, 37%) and disorder (10/49, 20%) were also noted. Disordered articulation errors were primarily /ɹ/ distortions (16/21, 76%) and interdental lisps (8/21, 38%). Speech inconsistency ranged from 0% to 94.7% inconsistent. Participants 36 and 51 presented with stuttering (severity rating

2 and 4, respectively). Only five participants did not have dysarthria or CAS. Participant 48 did not have a speech disorder but self-reported to have a speech disorder in childhood. Language and cognition scores were not available for participant 48. An additional eleven participants, whose English proficiency precluded assessment, were reported to have CAS by caregivers and were attending speech therapy.

Participants were 'usually' intelligible to caregivers on the ICS (mean=4.14, SD=0.79). Participants were 'sometimes' understood by extended family (mean=3.17, SD=1.01) and friends (mean=3.11, SD=1.06). Participants' intelligibility further decreased to 'rarely' understood by acquaintances (mean=2.88, SD=1.05) and strangers (mean=2.55, SD=1.10). Only five participants (Participants 70, 74, 59, 46, 89) were 'always' understood. There was no difference in average ICS scores between those with and without sleep disturbance (p=0.15), or by sex (p=0.08).

Connected speech tasks were acoustically analysed for articulation rate and phonation features (*n*=22). For further information see Supplementary Results 2.

DISCUSSION

Here we describe a large cohort of individuals with Kleefstra syndrome, encompassing a diverse representation of ages, genotypes, and countries. We have systematically characterized speech, language, and cognition to shed light on the complex communication profile of this rare genetic disorder. Language, cognition, and social responsiveness varied from average to severely impaired across the cohort, except for individuals with large (>1Mb) deletions. We identified no difference between receptive and expressive language, as has been previously reported in a

small cohort (*n*=8) (29). Speech disorders were striking and present in the absence of language or cognitive impairment. This indicates a core phenotype of speech disorder in Kleefstra syndrome, in the setting of a heterogenous neurobehavioural and medical profile.

CAS and paediatric dysarthria are rare motor speech disorders (2.4% and 3.4% of children with speech disorders in the general population, respectively) and greatly impact speech intelligibility (30). Our results thus confirm a core motor speech profile in Kleefstra syndrome (3). Motor speech disorders are also in the context of broader motor impairments, as is also seen in other genetic conditions associated with motor speech disorders (7-12). There are evidence-based interventions for CAS, and emerging evidence of therapies for paediatric dysarthria (31-33). Existing speech therapies have not been trialled in children with genetic conditions or cognitive impairment, underscoring the importance of future research in this area.

AAC was used by a range of participants, and not solely by those who were minimally verbal or with hearing impairment. In Kleefstra syndrome, AAC can be an important tool to support communication in the face of delayed speech and language milestones, severe speech disorders, hearing impairment, and communication skills affected by regression. AAC can also support quality of life and reduction of behaviours of concern (34, 35). A high proportion of caregivers believed AAC would hinder natural speech development. Clinicians should support caregivers to identify that AAC does not have negative implications for speech development and rather that AAC can develop fundamental communication skills (e.g., turn taking, symbolic communication) (36).

Lifelong access to therapy supports and services is imperative to meet the evolving needs of individuals with Kleefstra syndrome. Depending on the developmental stage and communication abilities, intervention requirements may vary from AAC intervention to CAS therapy. Additionally, tailored communication supports, such as literacy intervention and daily living communication skills, are essential during the school years, transitioning to adulthood and during periods of regression.

As has been previously reported in Kleefstra syndrome, sleep disturbance was common. Whilst sleep has been implicated in regression in some individuals, including in our study, sleep also plays an important role in language and cognitive development, behaviour, and mental health (37, 38). Individuals with Kleefstra syndrome would likely benefit from proactive referrals to sleep specialists, to promptly identify and address common sleep disturbances such as frequent waking.

In cross-sectional Kleefstra syndrome studies, it is challenging to assess genotype-phenotype correlations as speech and language skills change with age, and some individuals also present with regression. Despite the wide range of genotypes, no clear novel genotype-phenotype correlations were evident between different intragenic variants, or between locations and sizes of 9q34.3 deletions (<1Mb). However, we confirmed the previous observation of large deletions (>1Mb) being associated with a more severe phenotype. The small number of participants within each genotype group likely constrained the identification of potential genotype-phenotype correlations as well as the likely underrepresentation of participants with intragenic variants as this requires access to exome and genome sequencing which is less accessible than chromosomal microarray.

Caregiver reports provided insights into regression here, although we acknowledge the limitations of retrospective reporting. The underlying cause of regression in Kleefstra syndrome has not been identified. Apart from small case series, the neurological involvement contributing to regression has not been investigated (39). Our cohort had lower levels of regression than clinical reports (2), likely due to the relatively young age of our cohort, with typical Kleefstra syndrome regression often occurring in adolescence and adulthood (2). The regression reported in younger children may be reflective of early childhood regression observed in many neurodevelopmental conditions (40). This early regression is likely not unique to Kleefstra syndrome, which is distinct from the significant developmental regression occurring in several individuals in Kleefstra syndrome in adolescence and adulthood. Longitudinal, natural history studies are currently ongoing and are required to obtain a comprehensive understanding of regression incidence, its triggers, duration, and potential therapeutic interventions. Likewise larger cohorts are required to elucidate genotype-phenotype correlations and brain imaging studies could unravel the underlying neural mechanisms associated with regression, which have been investigated in ex vivo and animal models (41, 42).

In conclusion, this study offers significant insights into the speech, language, and cognition profile of individuals with Kleefstra syndrome, across ages and genotypes. The findings underscore the critical role of AAC in supporting communication, the importance of early, evidence-based intervention, and the necessity of lifelong access to tailored therapy supports in light of possible regression for some individuals.

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COMPETING INTERSTS

The authors declare no conflict of interest.

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DATA AVAILABILITY

The data from this study is available upon reasonable request to the corresponding author.

AUTHOR CONTRIBUTIONS

Conceptualisation (TK, ATM, LDM), data curation (LDM, ATM), formal analysis (LDM, DR, AV, DJA), funding acquisition (ATM, TK, LDM, AV, SS), investigation (LDM), methodology (ATM, LDM, MGPK, AV, FL, Amanda B, TK, IES), project administration (LDM, ATM), resources (LM, TK, ATM, MGPK, Arianne B, JK, EP, FL, SS, ZF, DM, HG), software (AV), supervision (IES, ATM), visualisation (LDM), writing manuscript (LDM, IES, ATM), review and editing manuscript (LDM, ATM, TK, IES, MGPK, DR, Arianne B, JK, EP, AV, FL, Amanda B, SS, ZF, DM, HG, DJA)

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ETHICS DECLARATION

Ethics approval was obtained from the Royal Children's Hospital Human Research Ethics Committee (HREC 37353A).

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FIGURE LEGENDS

Figure 1a Deletions of participants (*n*=41): the location and size of >1Mb and <1Mb deletions affecting *EHMT1* (NM_024757.5) in participants with Kleefstra syndrome using University of California Santa Cruz Genome Browser, and their Vineland Adaptive Behaviour Scales 2nd/3rd Edition (Vineland II/III) Adaptive Behaviour Composite (ABC) scores (*n*=38, normative mean=100, SD=15).

Figure 1b The locations of nonsense, frameshift and missense variants along *EHMT1* in participants with Kleefstra syndrome (*n*=42), and their Vineland Adaptive Behaviour Scales 2nd/3rd Edition (Vineland II/III) Adaptive Behaviour Composite (ABC) scores (*n*=36, normative mean=100, SD=15).

Figure 2 Expressive and receptive language skills (*n*=90) measured by the Vineland Adaptive Behaviour Scales 2nd/3rd Edition (Low 1 to 9, Moderately Low 10 to 12, Average >12). Social responsiveness (*n*=73) measured by the Social Responsiveness Scale 2nd Edition (<60 Within normal limits, 60-65 Mild, 66-75 Moderate, >75 Severe. Cognitive ability (*n*=78, Average >70, Mild IQ 55-70, Moderate IQ 35-55, Severe or below IQ <35). One participant with an intellectual disability without severity specified is not shown here. Dashed sections of the bar graphs indicate individuals with deletions >1Mb.

Figure 3 Previous and current augmentative and alternative communication (AAC) use in 103 participants with Kleefstra syndrome.

Figure 4 Speech disorder profiles in assessed, verbal participants with Kleefstra syndrome (*n*=49). 48/49 participants had one or more speech disorders; one participant did not have a speech disorder. The one participant without a speech disorder is not depicted here.

SUPPLEMENTARY MATERIAL

Supplementary Table 1 Acoustic speech battery methodology

Supplementary Table 2 Genotypes and demographic information in 103 individuals with Kleefstra syndrome

Supplementary Table 3 Additional health & medical features in 103 individuals with Kleefstra syndrome

Supplementary Table 4 Milestones by genotype in 102 individuals with Kleefstra syndrome

Supplementary Figure 1 Children's Communication Checklist 2nd Edition (CCC-2) subdomain scores

Supplementary Figure 2 Communication Matrix communication functions, behaviours and levels of communication

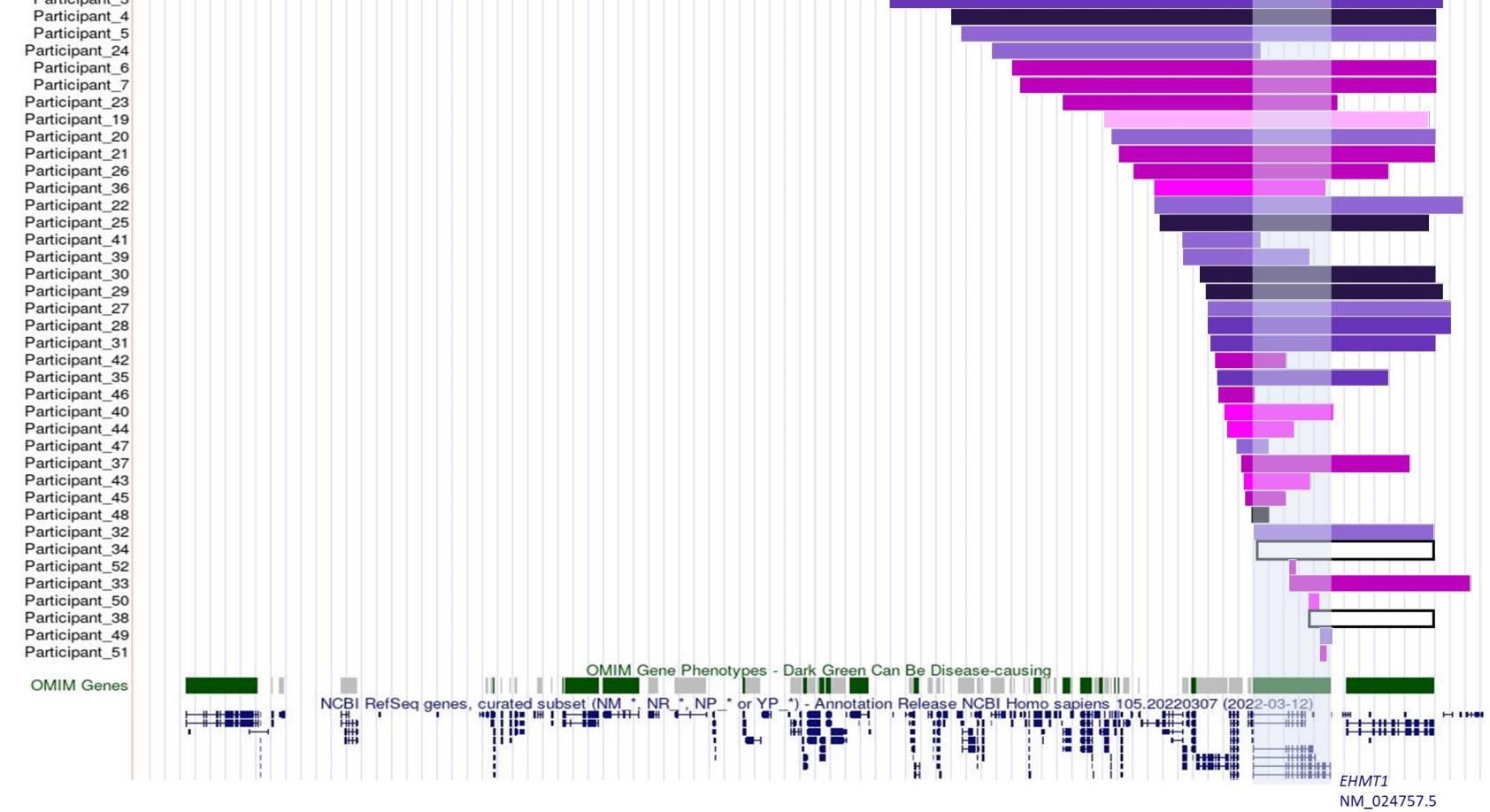
Supplementary Figure 3 Vineland Adaptive Behaviour Scores 2nd/3rd Edition domains

Supplementary Figure 4 Vineland Adaptive Behaviour Scales 2nd/3rd Edition adaptive behaviour composite scores, age and regression

Supplementary Figure 5 Social Responsiveness Scale 2nd Edition (SRS-2) T scores for 73 individuals with Kleefstra syndrome

Supplementary Results 1 Health, development and feeding

Supplementary Results 2 Acoustic speech analysis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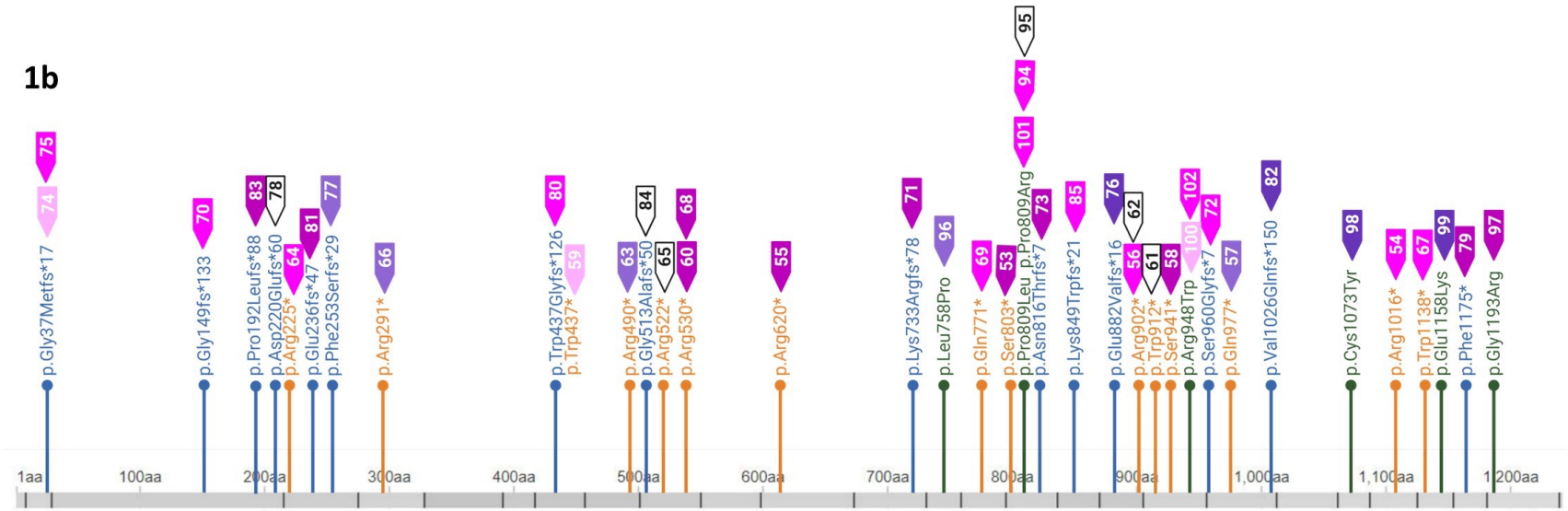
NM_024757.5


Vineland II/III ABC Score
>85
75-85
59-74
43-58
27-42
<27
Not assessed

1b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p.Arg225*
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p.Arg291*
p.Trp437Glyfs*126
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p.Gly513Alafs*50
p.Arg522*
p.Arg530*
p.Arg620*
p.Lys733Argfs*78
p.Leu758Pro
p.Gln771*
p.Ser803*
p.Pro809Leu
p.Pro809Arg
p.Asn816Thrfs*7
p.Lys849Trpfs*21
p.Glu882Valfs*16
p.Arg902*
p.Trp912*
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p.Arg948Trp
p.Ser960Glyfs*7
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p.Val1026Glnfs*150
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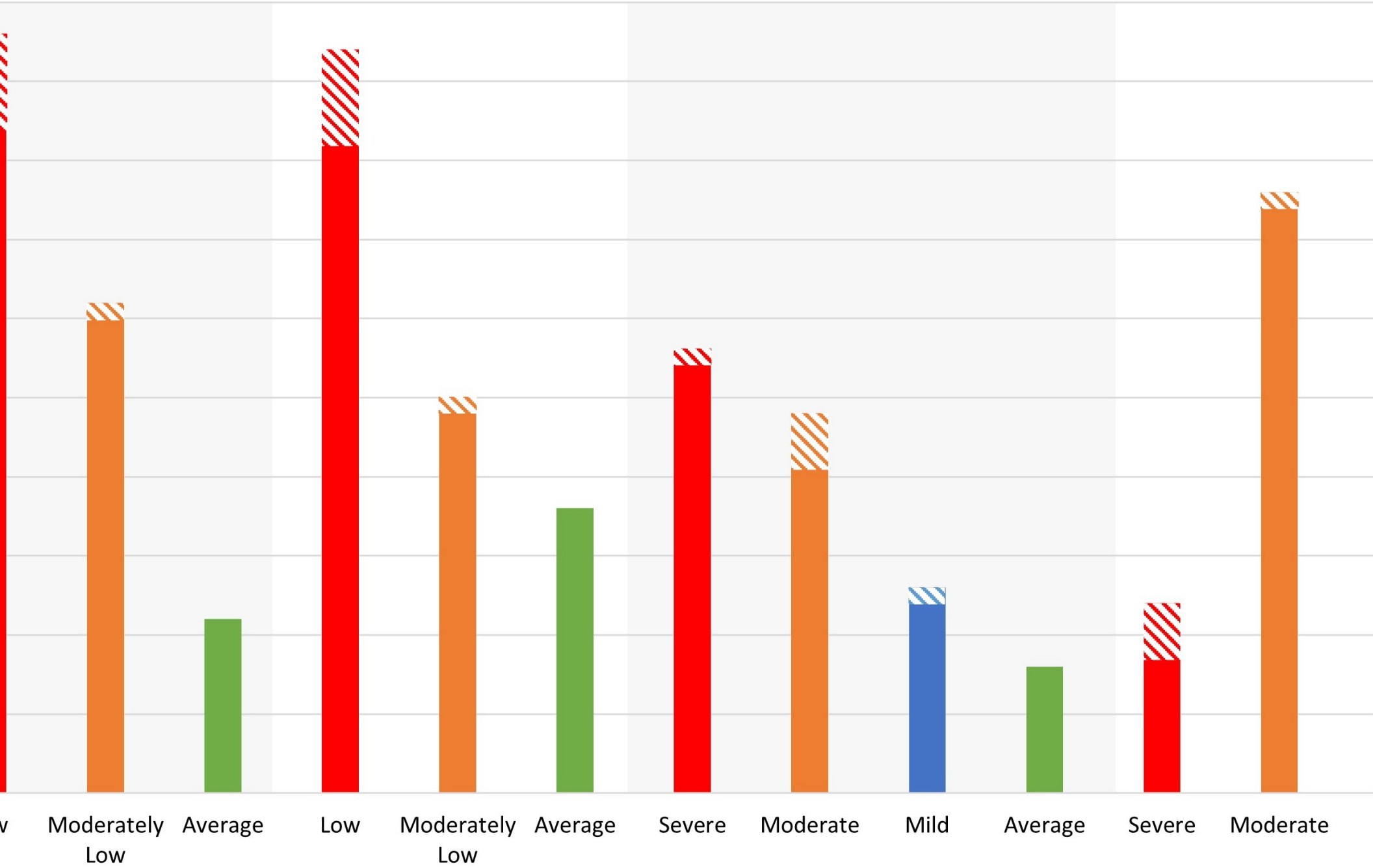
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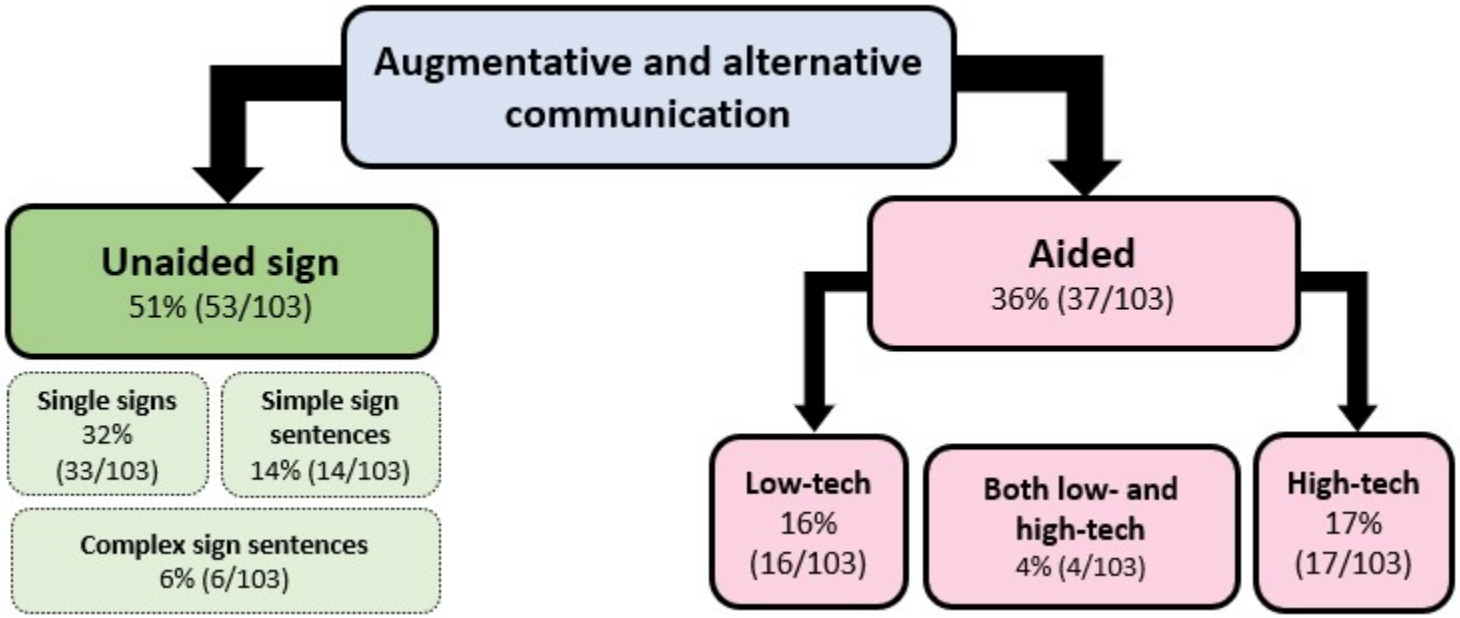
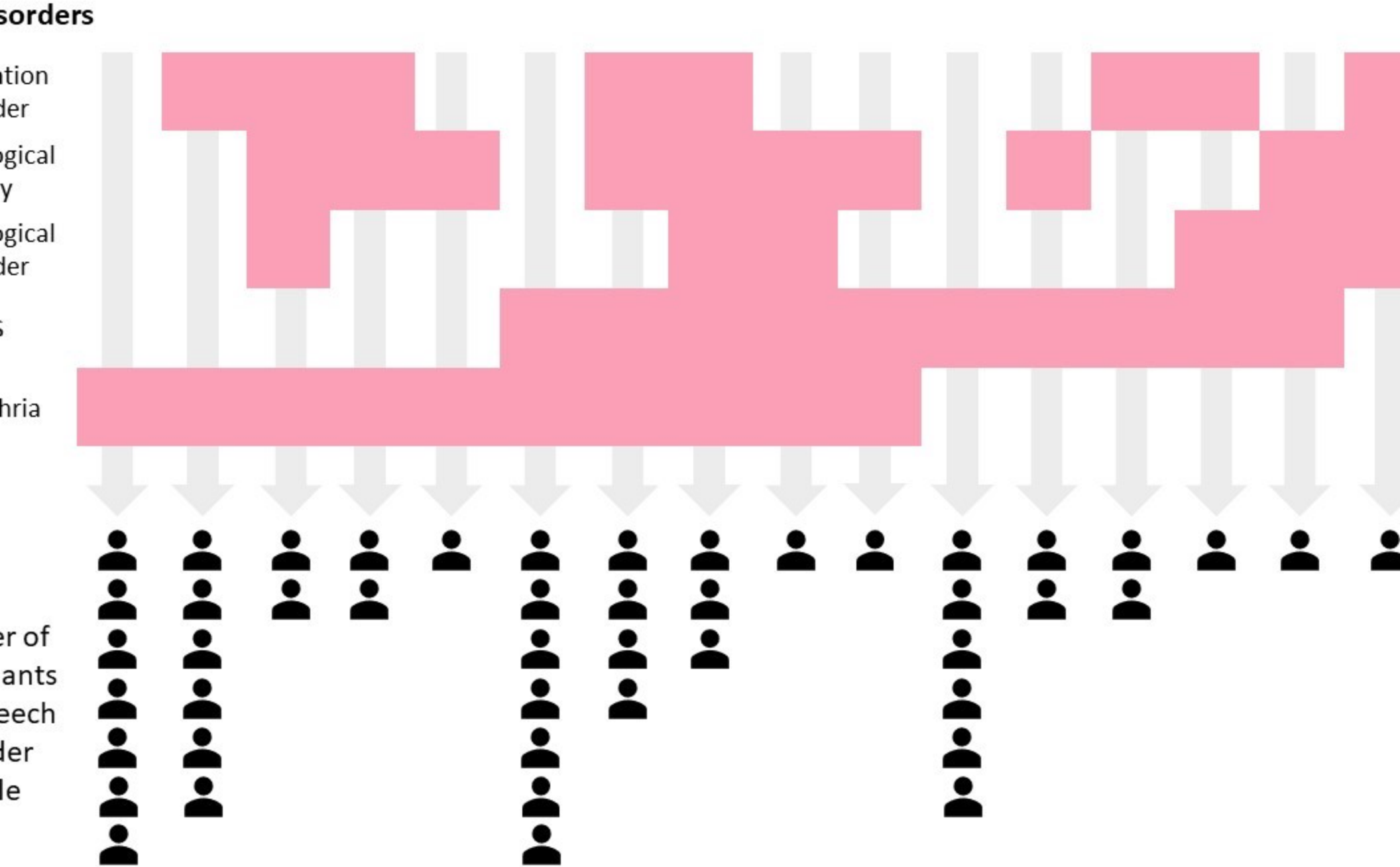

Augmentative and alternative communication
Unaided sign
51% (53/103)
Single signs
32%
(33/103)
Simple sign sentences
14% (14/103)
Complex sign sentences
6% (6/103)
Aided
36% (37/103)
Low-tech
16%
(16/103)
Both low- and high-tech
4% (4/103)
High-tech
17%
(17/103)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Supplementary Results 1

Health, development and feeding

Pregnancy complications included excessive amniotic fluid and reduced foetal movement (both 12/103, 12%). Birth complications included: emergency c-sections (12/103, 11.65%), assisted delivery with forceps or suction (8/103, 8%), and jaundice (8/103, 8%,). Some individuals required breathing support after birth (13/103, 13%) and resuscitation (2/103, 2%).

Of those with heart conditions (33/103, 32%), participants presented with electrocardiogram abnormalities (7/33), pulmonary stenosis (7/33), patent foramen ovale (4/33), cardiac malformations (3/33), and atrial (5/33) and ventral (3/33) septal defects. Gastrointestinal conditions (61/103, 59%) were frequent.

Several participants had dental conditions (59/103, 57%), which were described as complex orthodontics (21/59), too few (8/59) or too many (3/59,) teeth, slow loss of baby teeth (6/59) and delayed tooth eruption (5/59).

Other medical conditions included allergies (30/103, 29%), specifically to antibiotics (7/30), other medications (6/30), dairy (5/30), nuts (3/30), and other foods (6/30). Some participants also had eczema (21/103). Two participants had diabetes (Participants 15, 29). Musculoskeletal abnormalities (12/103, 12%) were also present. Urogenital conditions (14/103, 14%), included small (2/14) and enlarged kidneys (3/14).

Three-quarters of the cohort had undergone surgery (80/103, 78%) (Supplementary Table 3). Adenoidectomy (21/80) and tonsillectomy (11/80) were common surgeries, followed by tympanostomy tube insertion (34/80), ear drum (5/80) and hernia repair (8/80), and surgery for dental (8/80) and vision (8/80)

corrections. Male participants had surgery for hypospadias (2/40, 5%) and cryptorchidism (10/40, 25%).

Hypermetropia (37/103, 36%) was most frequent type of vision impairment, followed by strabismus (19/103, 18%), myopia (13/103, 13%) and a squint (10/103, 10%). Other forms of vision impairment were nystagmus (5/103, 5%), convergence insufficiency (3/103, 3%), cortical visual impairment (2/103, 2%). Several participants wore glasses (49/103, 48%).

Mixed (14/100, 14%), sensorineural (10/100, 10%) and conductive (11/100, 11%) hearing loss occurred at similar rates. Most participants had asymmetrical hearing loss (23/100, 23%). Mild (25-39dBHL, 16/100, 16%) and moderate (40-69dBHL, 17/100, 17%) hearing impairment was more common than severe (70-89dBHL, 2/100, 2%) and profound (>89dBHL, 1/100, 1%). Three participants had not had their hearing tested.

In addition to more common sleep disturbances, some caregivers also identified nocturnal vomiting (1/103, 1%), restless leg (1/103, 1%), and agitation at night (6/103, 6%).

Magnetic resonance imaging (MRI) findings (38/79) included cysts (6/38, 4 arachnoid cysts), hypoplasia of the corpus callosum (4/38), white matter abnormalities (5/38), and enlarged ventricles (4/38).

Supplementary Results 2

Acoustic speech analysis

Participants

Participants completed a monologue (6/22, 27%), or picture description tasks (5/22, 23%), or both tasks (11/22, 50%). For those who completed both tasks, measure averages of the picture description and monologue tasks were obtained. Participants spoke English (16/22, 73%), Spanish (2/22, 9%), French (2/22, 9%), Dutch (1/22, 5%), and Hungarian (1/22, 5%). Median age and sex can be found in the table below.

Eight participants had a deletion affecting *EHMT1* (8/22, 36%): 7/22 (32%) with <1Mb deletions and 1/22 had a deletion of unspecified size. Participants with *EHMT1* variants had nonsense, (5/11, 45%), frameshift (4/11, 36%), splice site (3/11, 27%), and missense (2/11, 18%) variants.

Motor speech disorders

In this study a speech pathologist perceptually diagnosed participants with childhood apraxia of speech (CAS, 2/22, 9%), dysarthria (8/22, 36%), and CAS and dysarthria (6/22, 27%). The caregivers of an additional two participants reported that their children had CAS and were receiving speech therapy, but English proficiency precluded direct assessment by a speech pathologist in this study. A further, four participants were also not assessed as they were not English-speaking.

Acoustic analysis results for individuals with CAS (*n*=4, 18%), dysarthria (*n*=8, 36%), and co-occurring CAS and dysarthria (*n*=6, 27%) can be found in the table below. There was no significant difference between the acoustic analysis variables in the three different speech disorder groups on Mann-Whitney tests ($p>0.05$).

Speech features

Acoustic analysis was conducted for speech rate and phonation measures. There was no clear pattern identified between Vineland II/III adaptive behaviour composite scores and acoustic analysis measures. Acoustic analysis results can also be found in the table and figures below.

Prosody

Prosodic measures included pause length, speech to pause ratio and articulation rate. Articulation rate is measured by calculating number of syllables per *x* time period in a connected speech sample (Vogel et al., 2017). Higher mean pause indicates more pausing (silence) during speech sample. Conversely, higher speech to pause ratio indicates more speech time relative to pauses, and higher articulation rate indicates faster speech rate. Consequently, individuals with motor speech disorders could have a higher mean pause rate, lower speech to pause ratio and lower articulation rate than speakers without motor speech disorders. Speech rate and mean pause length are both stable and sensitive markers of dysarthria (Vogel et al., 2011), and pauses can greatly impede on intelligibility (Bloch & Wilkinson, 2009; Vogel et al., 2017).

Phonation

Cepstral peak prominence and harmonic to noise ratio measured phonation quality (voice). Individuals with altered vocal quality (dysphonia) have lower cepstral peak prominence than speakers without dysphonia (Hidalgo-De la Guía et al., 2021). Cepstral peak prominence scores below 9.33 decibels for connected speech indicate dysphonia (Murton et al., 2020). A mean cepstral peak prominence of 17.46 (SD=2.50) indicating that dysphonia was not a core characteristic of this cohort.

Harmonic to noise ratio provides a ratio between period and non-periodic elements of speech, reflecting vocal hoarseness. A harmonic to noise ratio less than 20 is indicative of hoarseness (Boersma & Weenik, 2010). This cohort had an average harmonic to noise ratio of 15.86 decibels (SD=3.38), indicating that most of the group fell within the criteria for hoarse vocal quality.

Table. Acoustic speech measures from picture description and monologue speech tasks in individuals with Kleefstra syndrome (n=22)

	CAS (*n*=4)*	**Dysarthria** (*n*=8)	**CAS & dysarthria** (*n*=6)	**Average of group** (*n*=22)**
Sex	F=1, M=3	F=6, M=2	F=2, M=4	F=12, M=10
Median age (years, months)	7y11mo	15y8mo	17y7mo	14y3mo
Age range (years, months)	5y4mo-16y4mo	4y-26y10mo	13y8mo-26y1mo	4y-28y7mo
Average pause length (seconds)	0.63 ± 0.39	0.69 ± 0.46	0.93 ± 0.79	0.75 ± 0.52
Variability of pause length (seconds)	1.40 ± 0.48	1.52 ± 0.17	1.39 ± 0.37	1.46 ± 0.30
Speech to pause ratio (seconds)	0.67 ± 0.45	0.69 ± 0.60	0.76 ± 0.70	0.66 ± 0.53
Articulation rate (syllables across speech time)	4.07 ± 0.74	4.57 ± 1.96	3.73 ± 1.10	4.17 ± 1.46
Cepstral peak prominence (decibels)	16.45 ± 2.48	17.99 ± 2.20	18.36 ± 2.64	17.46 ± 2.50
Average harmonic to noise ratio (decibels)	17.86 ± 4.77	15.75 ± 3.65	15.65 ± 2.50	15.86 ± 3.38
Variability of harmonic to noise ratio (decibels)	0.41 ± 0.16	0.44 ± 0.12	0.40 ± 0.10	0.42 ± 0.11

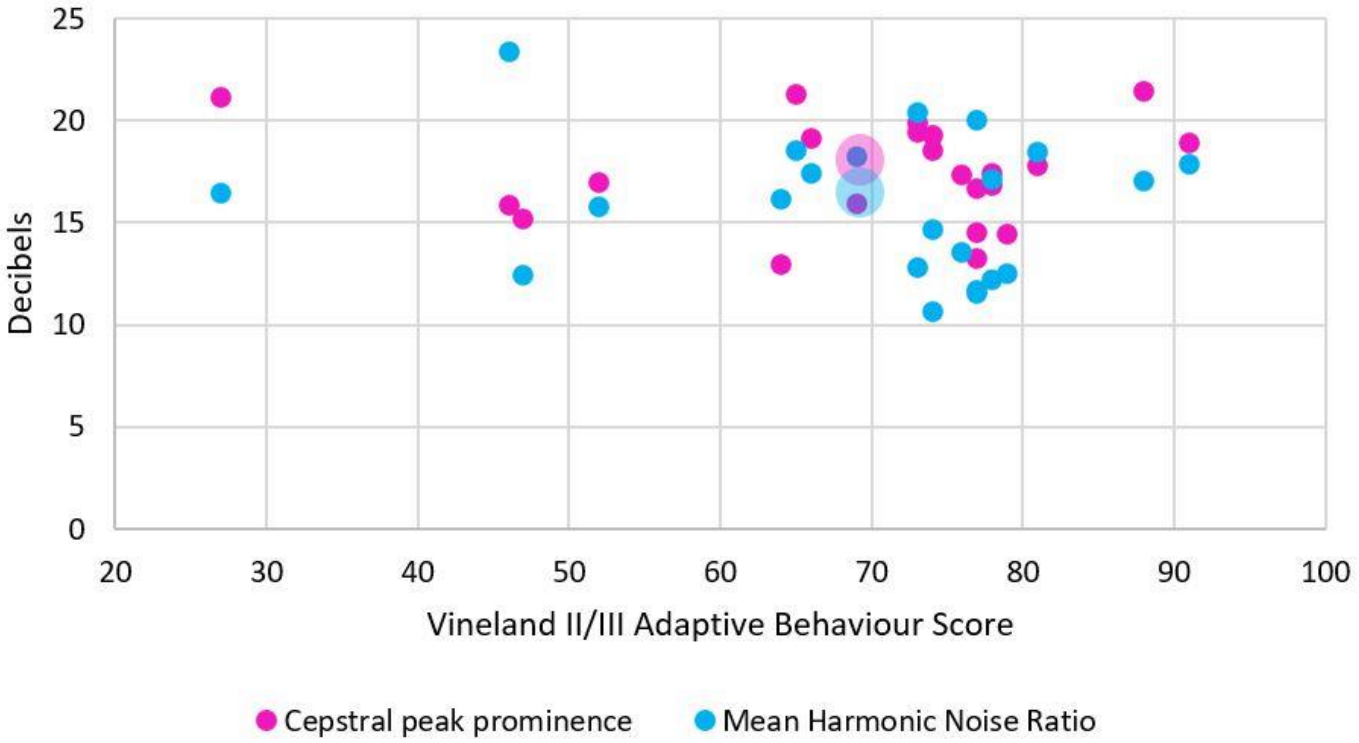
Values represent mean ± standard deviation

CAS = childhood apraxia of speech, F = Female, M = male, mo = months, y = years

*Two participants were reported by caregivers to have CAS and were seeing a speech pathologist, but were not assessed perceptually by a speech pathologist in this study as they were not English speaking.

**Four participants (3 females, 1 male) were not assessed perceptually by a speech pathologist in this study as they were not English speaking, and their caregivers did not report that they had CAS or dysarthria.

Cepstral peak prominence and harmonic to noise ratio from monologue and picture description tasks in individuals with Kleefstra syndrome (n=22)

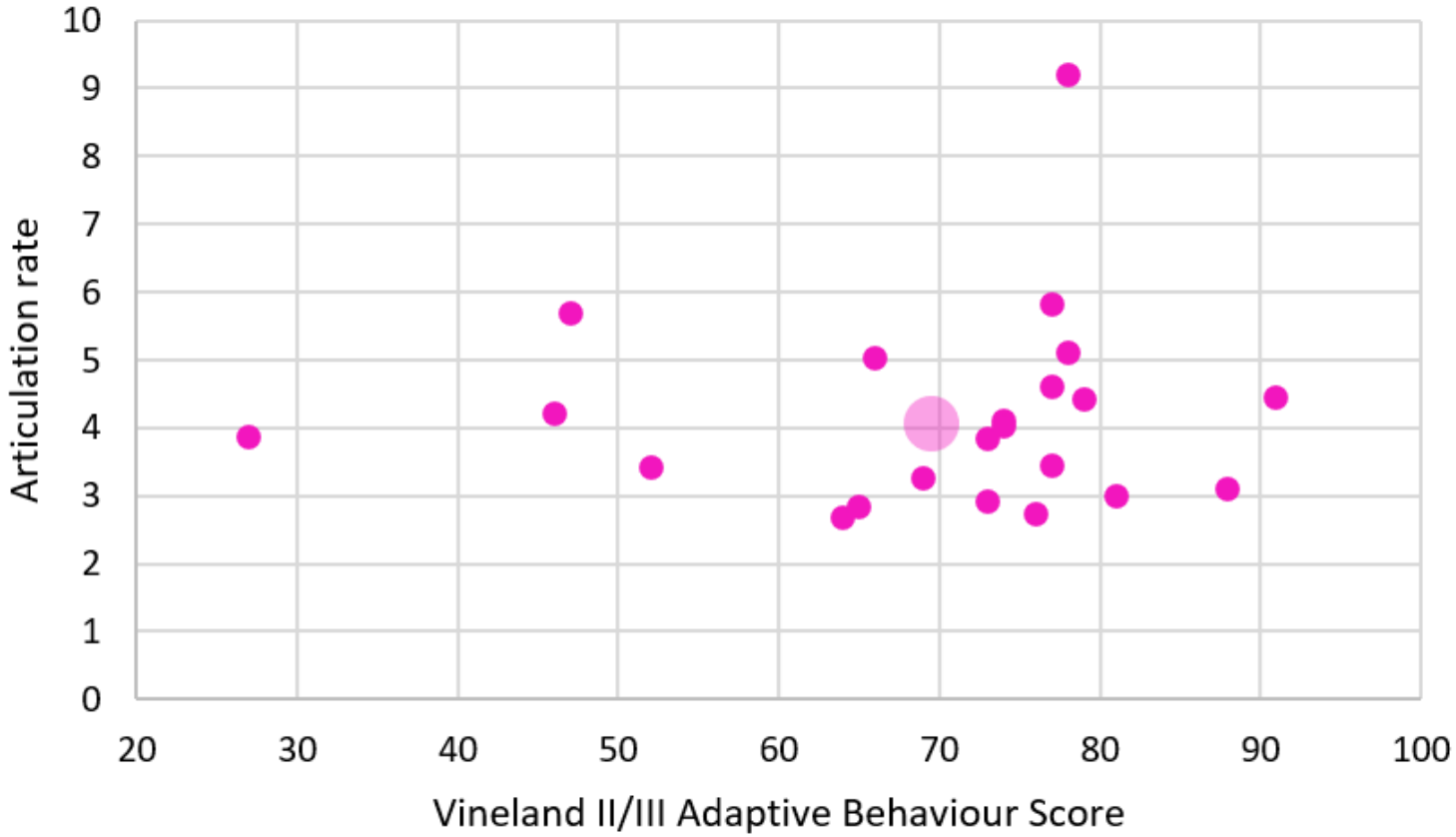


Spread of cepstral peak prominence and mean harmonic to noise ratio in individuals with Kleefstra syndrome (*n*=22) from picture description and monologue speech tasks, and Vineland II/III adaptive behaviour scores.

Cohort average cepstral peak prominence shown with transparent pink sphere. Cohort average harmonic to noise ratio shown with transparent blue sphere.

Lower cepstral peak prominence indicates dysphonia (voice abnormalities). Lower harmonic to noise ratio indicates higher hoarseness.

Articulation rate from monologue and picture description tasks in individuals with Kleefstra syndrome (n=22)

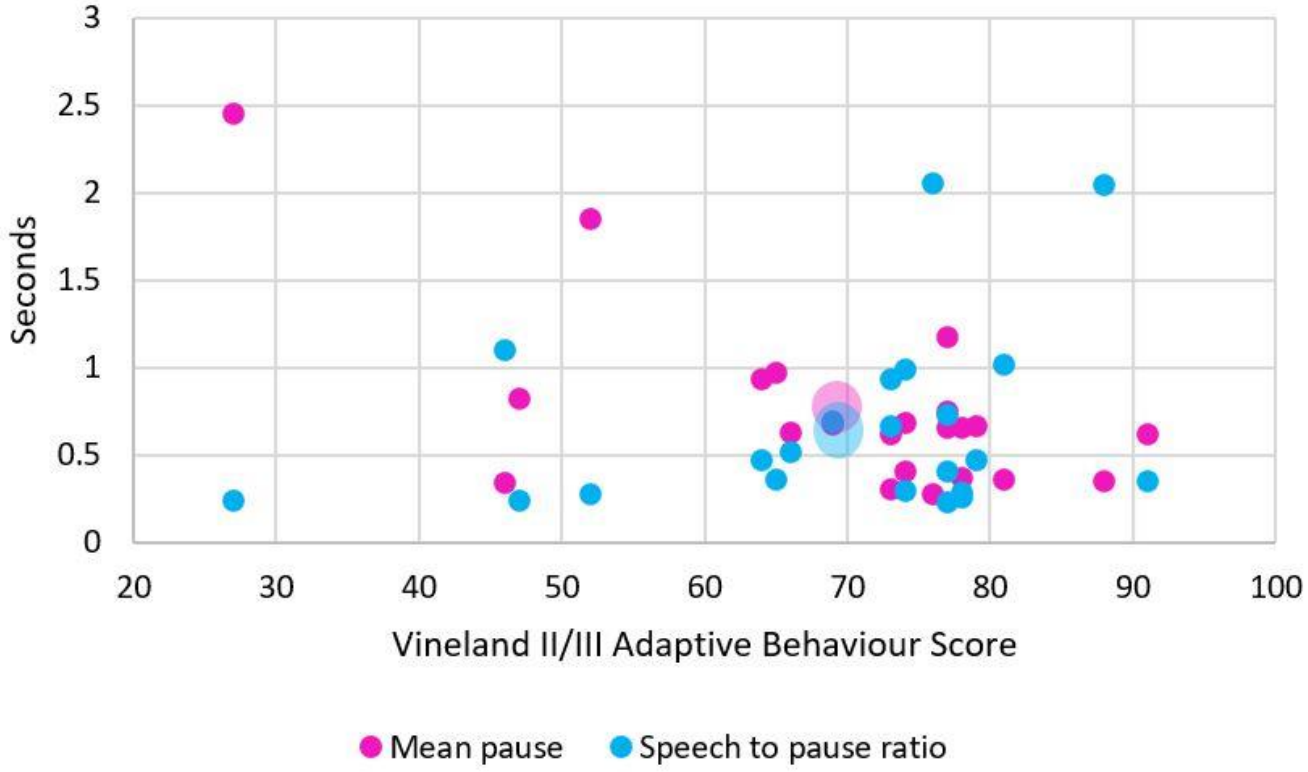


Spread of articulation rate (syllables per speech period) in individuals with Kleefstra syndrome (*n*=22) from picture description and monologue speech tasks, and Vineland II/III adaptive behaviour scores.

Cohort average articulation rate shown with transparent pink sphere.

Higher articulation rate indicates faster speech rate.

Mean pause and speech to pause ration from monologue and picture description tasks in individuals with Kleefstra syndrome (n=22)



Spread of mean pause and speech to pause noise ratio in individuals with Kleefstra syndrome (*n*=22) from picture description and monologue speech tasks, and Vineland II/III adaptive behaviour scores.

Cohort average mean pause shown with transparent pink sphere. Cohort average speech to pause noise ratio shown with transparent blue sphere.

Higher mean pause indicates more pausing during speech sample. Higher speech to pause ratio indicates more speech time relative to pauses.

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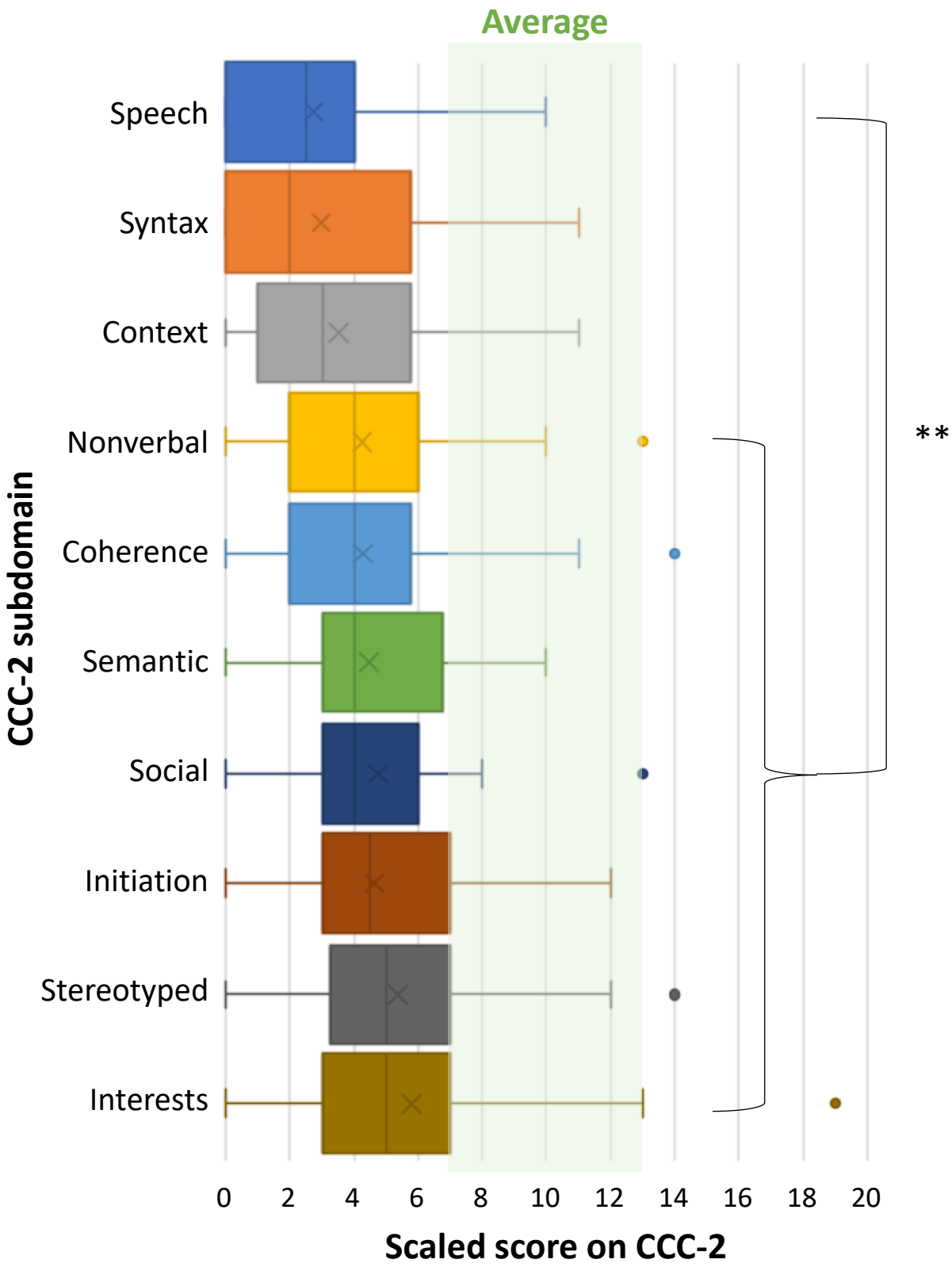
Supplementary Figure 1 Children's Communication Checklist 2nd Edition (CCC-2) subdomain scores



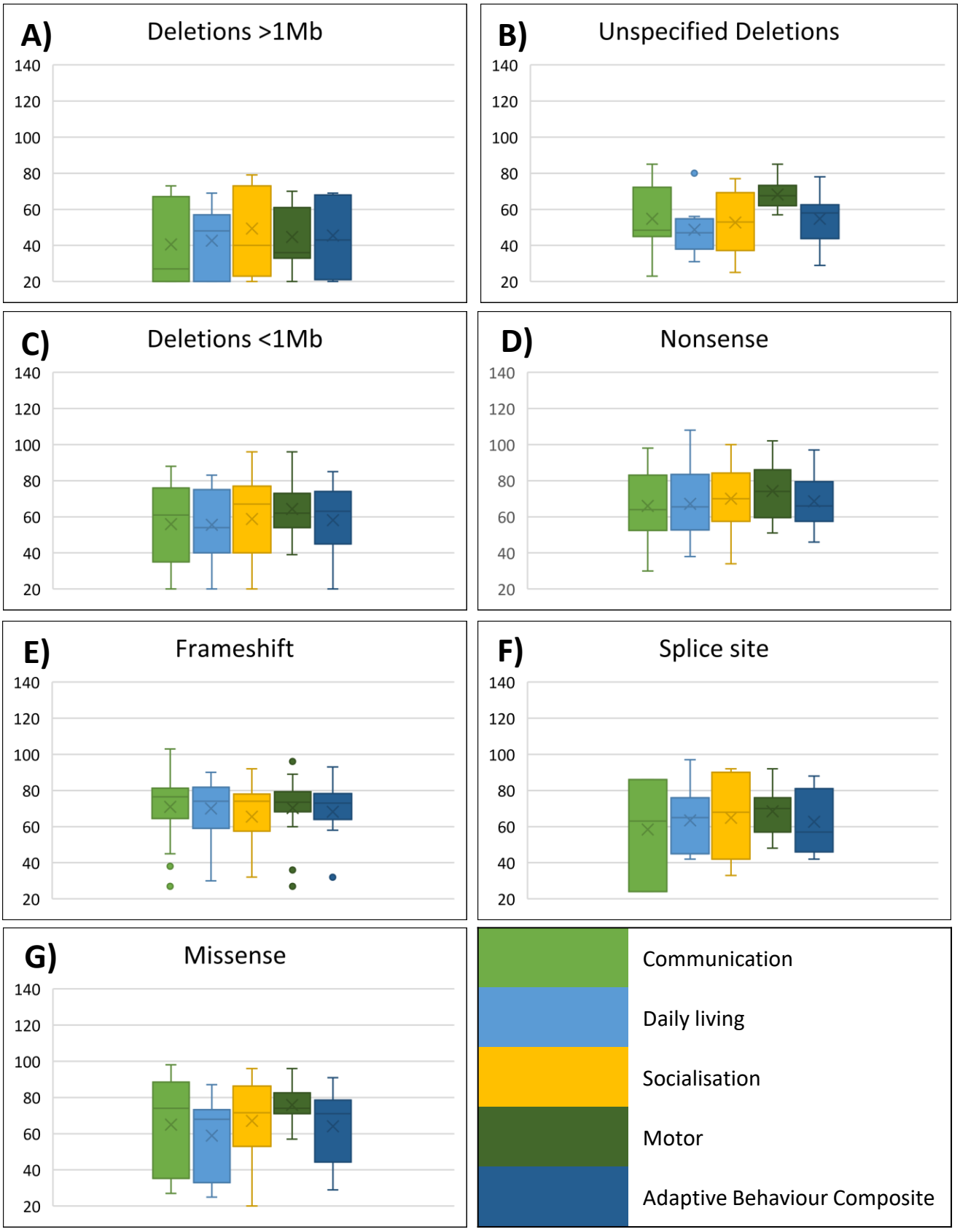
Individuals with Kleefstra syndrome (*n*=44). Normative mean = 10, SD = 3. Speech (mean = 2.75, SD = 2.80), syntax (mean = 2.98, SD = 3.57), context (mean = 3.52, SD = 2.78), nonverbal (mean = 4.25, SD = 2.87), coherence (mean = 4.27, SD = 2.86), semantic (mean = 4.47, SD = 2.57), social (mean = 4.75, SD = 3.14, inappropriate initiation (mean = 4.63, SD = 2.94), stereotyped (mean = 5.36, SD = 2.90), interests (mean = 5.80, SD = 3.51). ** = difference between subdomains and speech $p<0.05$. Mean = x, outliers = •.

Supplementary Figure 2 Communication Matrix communication functions, behaviours and levels of communication

<table>
<tr><th></th><th></th><th>Level</th><th>Refuse</th><th>Obtain</th><th>Social</th><th>Information</th></tr>
<tr><td rowspan="2">Pre-intentional communication</td><td></td><td>1: Preintentional behaviour</td><td>Expresses discomfort</td><td>Expresses comfort</td><td>Expresses interest in others</td><td rowspan="3"></td></tr>
<tr><td></td><td>2: Intentional behaviour</td><td>Protests</td><td>Continues an action
Obtains more of something</td><td>Attracts attention</td></tr>
<tr><td rowspan="5">Intentional communication</td><td rowspan="2">Pre-symbolic</td><td>3: Unconventional</td><td rowspan="5">Refuses or rejects</td><td rowspan="2">Requests more of an action
Requests new actions
Requests more of an object
Makes choices
Requests new objects</td><td>Requests attention
Shows affection</td></tr>
<tr><td>4: Conventional</td><td rowspan="3">Greets people
Offers or shares
Directs attention to something
Polite social forms</td><td>Answers yes/no
Asks questions</td></tr>
<tr><td rowspan="3">Symbolic</td><td>5: Concrete symbols</td><td rowspan="3">Requests absent objects</td><td rowspan="3">Names things or people
Makes comments</td></tr>
<tr><td>6: Abstract symbols</td></tr>
<tr><td>7: Language Combining symbols, signs, words</td></tr>
</table>

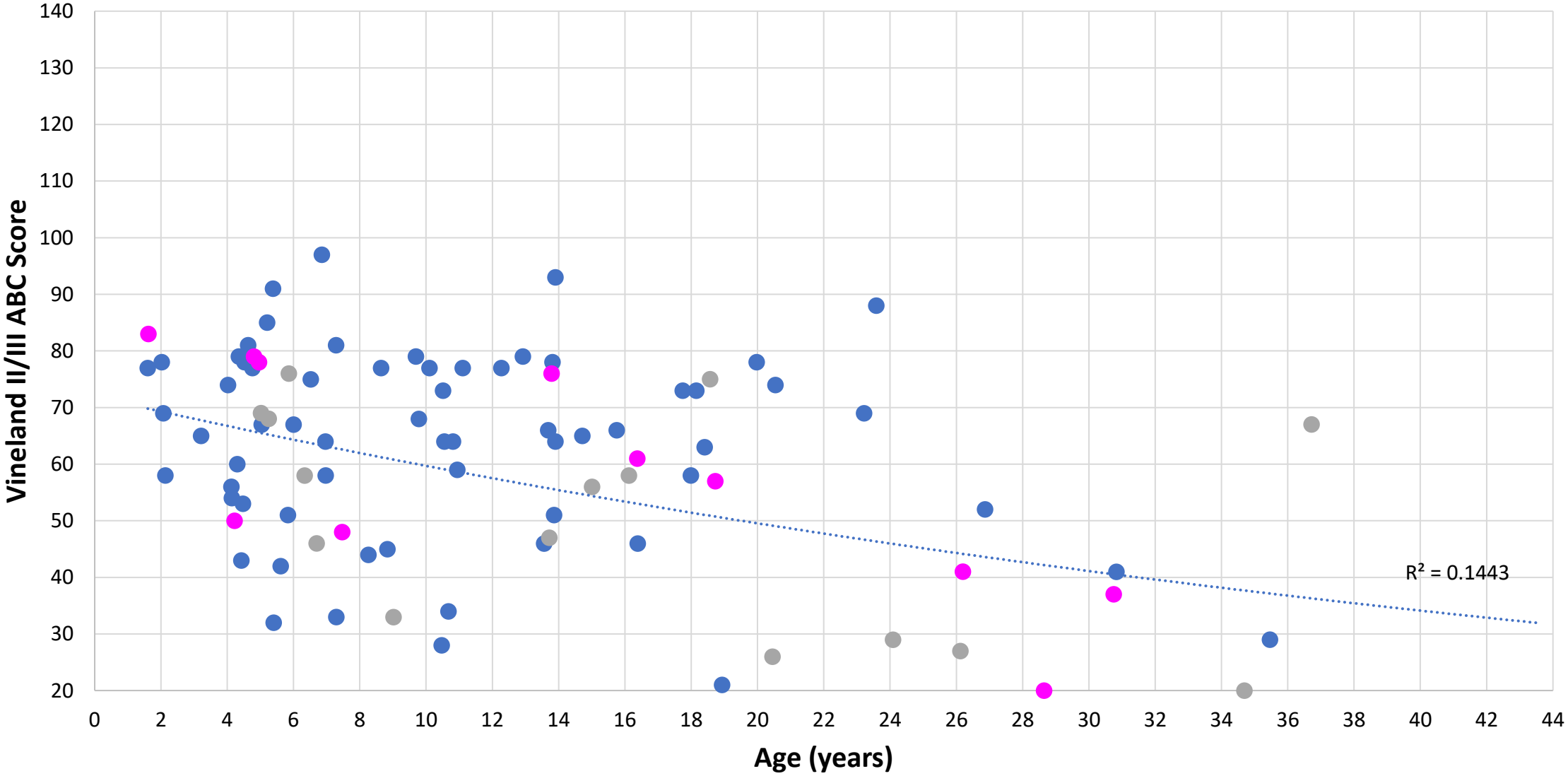
Communication matrix assesses communication behaviours across four communication functions (refuse, obtain, social, information). Communication behaviours can be categorised into levels of communication (level 1 to level 7)

Supplementary Figure 3 Vineland Adaptive Behaviour Scores 2nd/3rd Edition domains



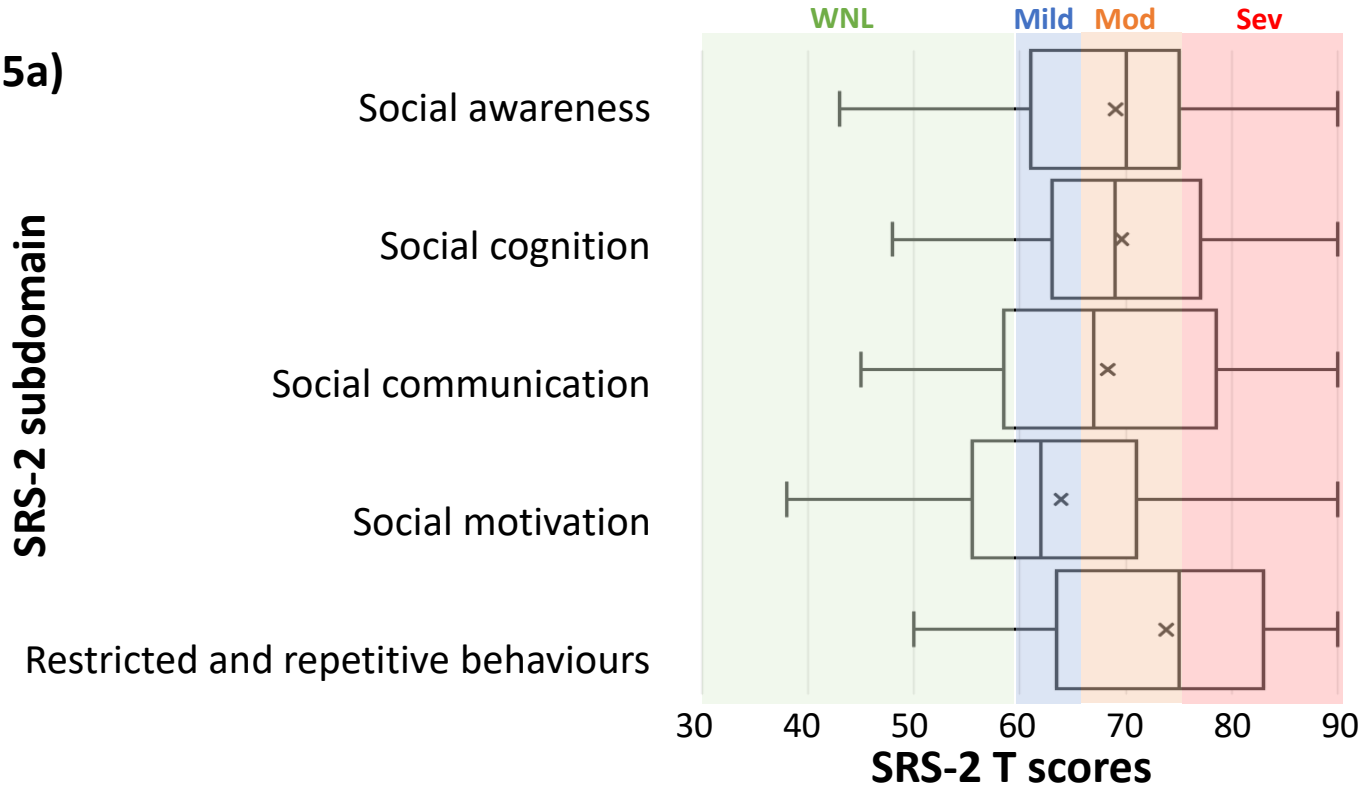
Domain scores from the Vineland Adaptive Behaviour Scores 2nd/3rd Edition in a cohort of individuals with Kleefstra Syndrome (*n*=90, scores 20-70 low, 81-85 moderately low, >85 average or above).
Deletions >1Mb *n*=7, unspecified deletions n=8, deletions <1Mb *n*=31, nonsense *n*=14, frameshift *n*=14, splice site *n*=7, missense *n*=8. Scores for 1 participant with balanced translocation are not shown here.
Mean = x, outliers = •

Supplementary Figure 4 Vineland Adaptive Behaviour Scales 2nd/3rd Edition adaptive behaviour composite scores, age and regression

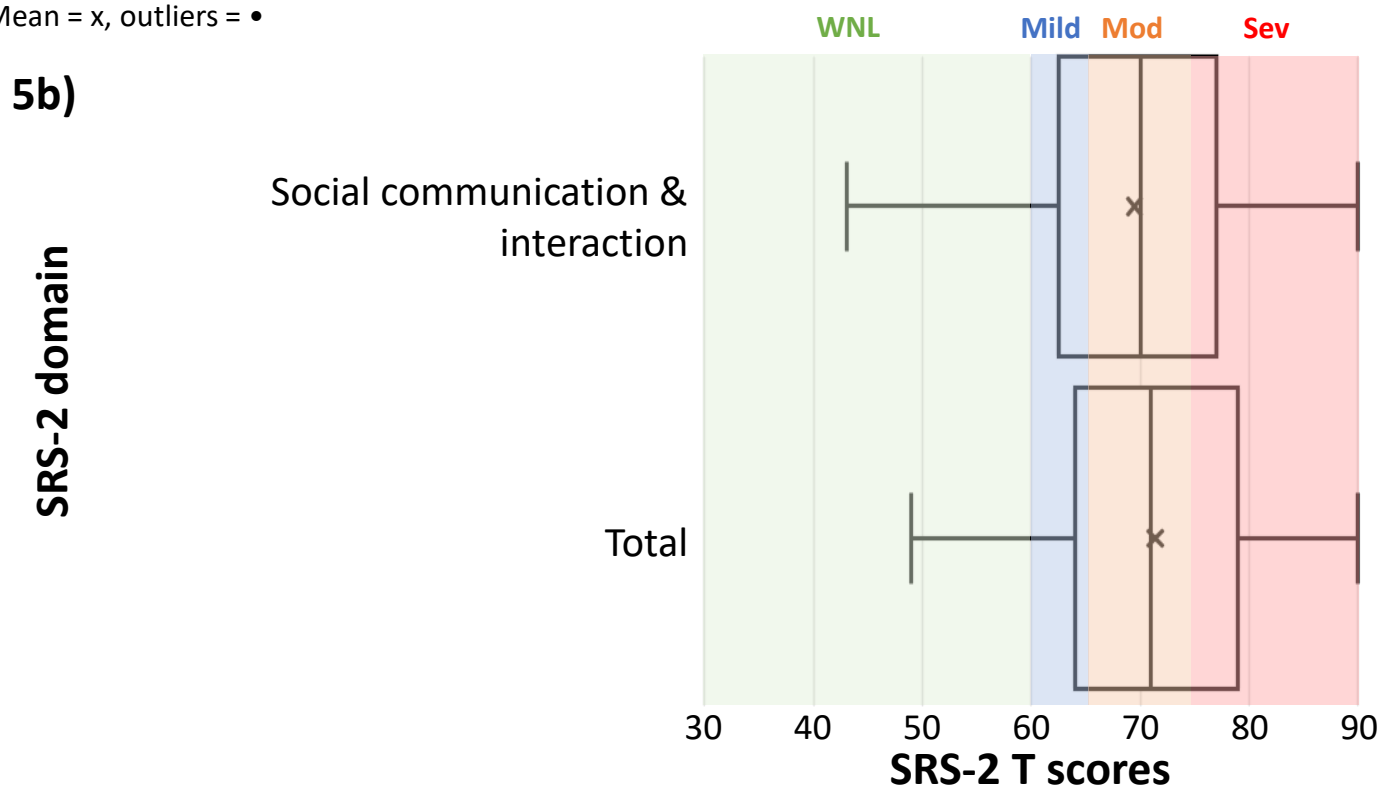


Age (years) and Vineland Adaptive Behaviour Scale 2nd / 3rd Edition (Vineland II/III) Adaptive Behaviour Composite (ABC) Scores (scores 20-70 low, 81-85 moderately low, >85 average or above) of participants with Kleefstra Syndrome (*n*=90). **Pink** markers indicate participants who have reported regression (*n*=11) by caregivers on the Development and Neurobehavioural Regression (DANR) questionnaire, **grey** markers indicate participants who did not complete the DANR (*n*=15), and **blue** markers indicate participants who were reported to have no regression on the DANR (*n*=64). 5 participants completed the DANR but did not complete the Vineland II/III.

Supplementary Figure 5 Social Responsiveness Scale 2nd Edition (SRS-2) T scores for 73 individuals with Kleefstra syndrome



<60 within normal limits, 60-65 mild, 66-75 moderate, >75 severe.
Social awareness (mean = 69, SD = 10.76), social cognition (mean = 69.63, SD = 9.91), social communication (mean = 68.30, SD = 11.24), social motivation (mean = 63.89, SD = 21.02), restricted and repetitive behaviours (mean = 73.82, SD = 11.57).
Mean = x, outliers = •



<60 within normal limits, 60-65 mild, 66-75 moderate, >75 severe.
Social communication and interaction (total of social subdomains; mean = 69.47, SD = 11.04), social responsiveness total (total of all subdomains; mean = 71.41, SD = 10.35)
Mean = x, outliers = •

Supplementary Table 1 Acoustic speech battery methodology

Speech subsystem	Measure	Stimuli	Task
Prosody	Speech rate	Connected speech	Monologue and picture description
	Mean silence length	Connected speech	Monologue and picture description
	Variation of silence length	Connected speech	Monologue and picture description
	Percent silence	Connected speech	Monologue and picture description
Phonation	Cepstral peak prominence	Connected speech	Monologue and picture description
	Harmonics to noise ratio	Connected speech	Monologue and picture description

Supplementary Table 2 Genotypes and demographic information of 103 individuals with Kleefstra syndrome

ID	Sex	Age at assessment (Age range in yrs)	Country	g.DNA (GRCh37)	Inheritance	Pathogenicity
Deletions†						
>1Mb deletion						
1	M	3-5	US	chr9:g.(138347823_140893796)x1	De novo	Pathogenic
2	M	18-20	AUS	chr9:g.(139132184_141073875)x1	De novo	Pathogenic
3	F	9-11	SPAN	chr9:g.(139502842_141045981)x1	De novo	Pathogenic
4	F	27-29	US	chr9:g.(139674488_141020389)x1	De novo	Pathogenic
5	F	6-8	UK	chr9:g.(139703427_141018984)x1	De novo	Pathogenic
6	F	0-2	US	chr9:g.(139840430_141020389)x1	De novo	Pathogenic
7	M	9-11	US	chr9:g.(139872006_141019079)x1	De novo	Pathogenic
Unspecified deletion						
8	F	6-8	NL	9q34.3 deletion	Unknown	Pathogenic
9	F	15-17	US		De novo	
10	M	12-14	CAN		Unknown	
11	F	6-8	SPAN		De novo	
12	F	0-2	BULG		Unknown	
13	F	0-2	MEX		Unknown	
14	F	15-17	UK		Unknown	
15	M	21-23	NL		De novo	
16	M	33-35	US		Unknown	
17	M	24-26	NZ		Unknown	
18	M	24-26	US		Unknown	
<1Mb deletion						
19	M	3-5	US	chr9:g.(140099198_141005514)x1	De novo	Pathogenic
20	M	6-8	US	chr9:g.(140120700_141018984)x1	De novo	Pathogenic
21	F	3-5	US	chr9:g.(140140868_141020389)x1	De novo	Pathogenic
22	F	15-17	SPAN	chr9:g.(140240417_141102518)x1	Unknown	Pathogenic

23	F	3-5	PORT	chr9:g.(139987088_140741154)x1	De novo	Pathogenic
24	M	0-2	US	chr9:g.(139784913_140533414)x1	De novo	Pathogenic
25	M	18-20	COL	chr9:g.(140253734_140994780)x1	Unknown	Pathogenic
26	F	18-20	US	chr9:g.(140187786_140894343)x1	De novo	Pathogenic
27	M	3-5	UK	chr9:g.(140389536_141066496)x1	De novo	Pathogenic
28	F	30-32	US	chr9:g.(140390614_141064741)x1	De novo	Pathogenic
29	M	33-35	AUS	chr9:g.(140382705_141044489)x1	De novo	Pathogenic
30	M	24-26	US	chr9:g.(140366594_141020389)x1	Unknown	Pathogenic
31	F	9-11	SWED	chr9:g.(140401671_141020389)x1	De novo	Pathogenic
32	F	3-5	UK	chr9:g.(140515592_141018976)x1	De novo	Pathogenic
33	F	15-17	US	chr9:g.(140618901_141122085)x1	De novo	Pathogenic
34	F	9-11	COL	chr9:g.(140527202_141019079)x1	De novo	Pathogenic
35	F	6-8	ARG	chr9:g.(140418418_140893129)x1	Unknown	Pathogenic
36	F	9-11	SA	chr9:g.(140240414_140714465)x1	De novo	Pathogenic
37	F	3-5	BRAZ	chr9:g.(140482479_140954147)x1	De novo	Pathogenic
38	M	6-8	US	chr9:g.(140670375_141020389)x1	De novo	Pathogenic
39	F	3-5	AUS	chr9:g.(140322576_140659890)x1	De novo	Pathogenic
40	F	3-5	BEL	chr9:g.(140435487_140738221)x1	De novo	Pathogenic
41	M	6-8	US	chr9:g.(140320686_140527261)x1	De novo	Pathogenic
42	M	3-5	AUS	chr9:(140408189_140610042)x1 [0.7]	De novo	Pathogenic
43	M	6-8	US	chr9:g.(140489539_140676934)x1	Unknown	Pathogenic
44	F	18-20	COL	chr9:g.(140441805_140622664)x1	De novo	Pathogenic
45	M	3-5	CAN	chr9:g.(140493728_140603975)x1	De novo	Pathogenic
46	F	15-17	SPAN	chr9:g.(140419439_140519724)x1	De novo	Pathogenic
47	M	12-14	IRL	chr9:g.(140469021_140550967)x1	De novo	Pathogenic
48	M	42-44	US	chr9:g.(140511220_140558134)x1	Unknown	Pathogenic
49	F	12-14	DK	chr9:g.(140703393_140734243)x1	Unknown	Pathogenic
50	F	12-14	US	chr9:g.(140667619_140697333)x1	De novo	Pathogenic
51	M	18-20	UK	chr9:g.(140703416_140715310)x1	De novo	Pathogenic
52	F	9-11	GER	chr9:(140618871_140627749)x1	Unknown	Pathogenic

Point variants‡							
ID		**Age at assessment (yrs)**	**Country**	**c.DNA (**NM_024757.5**)**	**Protein**	**Inheritance**	**Pathogenicity**
Nonsense							
53	F	15-17	US	c.2408C>G	p.(Ser803*)	De novo	Pathogenic
54	M	3-5	SPAN	c.3046C>T	p.(Arg1016*)	De novo	Pathogenic
55	M	12-14	AUS	c.1858C>T	p.(Arg620*)	Unknown	Pathogenic
56	F	3-5	AUS	c.2704C>T	p.(Arg902*)	Unknown	Pathogenic
57	F	12-14	NL	c.2929C>T	P.(Gln977*)	De novo	Pathogenic
58	F	9-11	NL	c.2822C>A	p.(Ser941*)	De novo	Pathogenic
59	F	6-8	SLOV	c.1311G>A	p.(Trp437*)	De novo	Pathogenic
60	M	3-5	SLOV	c.1588C>T	p.(Arg530*)	De novo	Pathogenic
61	F	15-17	SPAN	c.2735G>A	p.(Trp912*)	De novo	Pathogenic
62	M	9-11	BRAZ	c.2704C>T	p.(Arg902*)	Unknown	Pathogenic
63	F	3-5	GER	c.1468C>T	p.(Arg490*)	De novo	Pathogenic
64	F	12-14	US	c.673C>T	p.(Arg225*)	De novo	Pathogenic
65	F	3-5	SEB	c.1566C>A	p.(Cys522*)	De novo	Pathogenic
66	F	12-14	UK	c.871C>T	p.(Arg291*)	De novo	Pathogenic
67	F	18-20	HUN	c.3413G>A	p.(Trp1138*)	De novo	Pathogenic
68	M	9-11	US	c.1588C>T	p.(Arg530*)	De novo	Pathogenic
69	M	0-2	US	c.2311C>T	p.(Gln771*)	De novo	Pathogenic
Frameshift							
70	F	9-11	UK	c.444delT	p.(Gly149Alafs*133)	De novo	Pathogenic
71	F	6-8	GER	c.2198del	p.(Lys733Argfs*78)	De novo	Pathogenic
72	F	9-11	US	c.2877_2880delTTCT	p.(Ser960Glyfs*7)	De novo	Pathogenic
73	F	21-23	US	c.2447delA	p.(Asn816Thrfs*7)	De novo	Pathogenic
74	F	12-14	US	c.109delGinsAT	p.(Gly37Metfs*17)	Unknown (75 sister)	Likely pathogenic
75	F	12-14	US	c.109delGinsAT	p.(Gly37Metfs*17)	Unknown (74 sister)	Likely pathogenic

76	F	3-5	BEL	c.2645_2646delAG	p.(Glu882Valfs*16)	De novo	Pathogenic	
77	M	6-8	US	c.756delC	p.(Phe253Serfs*29)	De novo	Pathogenic	
78	F	3-5	BRAZ	c.656_663delinsA	p.(Asp220glufs*60)	De novo	Pathogenic	
79	M	18-20	SPAN	c.3524_3525delTT	p.(Phe1175*)	De novo	Pathogenic	
80	F	3-5	EST	c.1308delC	p.(Trp437Glyfs*126)	Unknown	Pathogenic	
81	F	12-14	UK	c.704_705dupAG	p.(Glu236fs*47)	Unknown	Pathogenic	
82	F	9-11	AUS	c.3072_3073delCT	p.(Val1026Glnfs*150)	De novo	Pathogenic	
83	M	9-11	GER	c.575_581delCGGCCCC	p.(Pro192Leufs*88)	De novo	Pathogenic	
84	M	3-5	BRAZ	c.1538delG	p.(Gly513Alafs*50)	De novo	Pathogenic	
85	F	3-5	CROAT	c.2545_2552delinsTGG	p.(Lys849Trpfs*21)	Unknown	Pathogenic	
Splice site								
86	M	0-2	US	c.2505+1G>A	p.?	Unknown	Likely Pathogenic	
87	M	6-8	CAN	c.2867+5G>A	p.?	De novo	Likely Pathogenic	
88	F	18-20	AUS	c.3540+2T>C	p.?	De novo	Likely Pathogenic	
89	F	15-17	FR	c.3180+1G>A	p.?	De novo	Likely Pathogenic	
90	F	21-23	US	c.3459C>T	p.?	Unknown	Pathogenic	
91	M	3-5	SWITZ	c.3540G>A	p.?	De novo	Likely Pathogenic	
92	F	3-5	US	c.3181-1G>T	p.?	Unknown	Likely Pathogenic	
93	M	6-8	AUS	c.3459C>T	p.?	De novo	Likely Pathogenic	
Missense								**Domain**
94	F	3-5	UK	c.2426C>T	p.(Pro809Leu)	De novo	Pathogenic	ANKR
95	F	6-8	US	c.2426C>G	p.(Pro809Arg)	De novo	Likely Pathogenic	ANKR
96	M	3-5	NL	c.2273T>C	p.(Leu758Pro)	Paternal mosaic (5% in blood)	Pathogenic	ANKR
97	M	36-38	US	c.3577G>A	p.(Gly1193Arg)	De novo	Likely Pathogenic	SET
98	F	24-26	UK	c.3218G>A	p.(Cys1073Tyr)	De novo	Likely Pathogenic	Pre-SET

99	F	30-32	SPAN	c.3472G>A	p.(Glu1158Lys)	De novo	Likely Pathogenic	SET
100	M	3-5	AUS	c.2842C>T	p.(Arg948Trp)	De novo	Likely Pathogenic	ANKR
101	F	3-5	AUS	c.2426C>T	p.(Pro809Leu)	De novo	Likely Pathogenic	ANKR
102	F	6-8	AUS	c.2842C>T	p.(Arg948Trp)	De novo	Likely Pathogenic	ANKR
Heterozygous balanced translocation†								
103	F	6-8	SPAN	46,XX,t(9;15)(q34.1;q13) seq[GRCh19] g.[chr9:pter_cen_140635745::chr15:33841983_qter] g.[chr15:pter_cen_33841977::chr9:140635750_qter]		De novo	Pathogenic	

† = Hg19, NM_024757.5

‡ = unspecified due to diagnosis made by fluorescence in situ hybridisation (FISH, *n*=3), or deletion details simply not being available (*n*=8)

ARG = Argentina, AUS = Australia, BEL = Belgium, BRAZ = Brazil, Bulg = Bulgaria, CAN = Canada, COL = Colombia, CROAT = Croatia, DK = Denmark, EST = Estonia, F = female, FR = France, GER = German, HUN = Hungary, IRL = Ireland, M = male, MEX = Mexico, NL = The Netherlands, PORT = Portugal, SPAN = SPAIN, SWED = Sweden, SWITZ = Switzerland, UK = United Kingdom, US = United States, yrs = years

Supplementary Table 3 Additional health & medical features in 103 individuals with Kleefstra syndrome

ID	Pregnancy	Birth	Musculoskeletal/ Movement	Cardiac	Urogenital	Epilepsy	MRI/CT	Surgery	Other	Vision impairment	Hearing loss symmetry	Hearing loss type	Hearing loss severity[‡]	Cognitive ability	Regression[†]
1	-	↓ oxygen, jaundice	DCD	Bicuspid aortic valve	-	Lennox Gastaut, tonic, grand mal, focal, generalized	Vol loss in the periventricular WM, particularly posteriorly. Mild vol loss within brainstem, especially in midbrain	G/PE tube	Infantile spasms, many various seizure types	CVI	Asymmetrical	Mixed	Mod	NA	-
2	↑ nuchal fold thickness, abnormal ultrasound results	Long labour, ↓ oxygen	-	TOF	-	-	NA	Grommets, adenoids, tonsillectomy, dental, heart surgery	-	-	Asymmetrical	Mixed	Mod	Sev	-
3	↑ nuchal fold thickness, ↓ amniotic fluid	Failed hearing test	-	Unspecified	-	-	Hyperintensity T2 & LFAIR of bilateral peri atrial deep sagittal, coronal & axial plane without alterations	-	Bronchial hyperactivity	Hypermetropia	Asymmetrical	Sensorineural	Sev	NA	NA
4	Break through bleeding	↓ APGAR scores, difficulty	-	-	-	Grand mal	Unspecified	Umbilical hernia operation	Hypothyroidism, mild gut	-	Asymmetrical	Conductive	Mod	Sev	+

	in first trimester, ↓ progesterone, ↓ weight gain/belly circumference, ↓ foetal movement, abnormal ultrasound results	regulating temperature						, tear ducts blocked, Brown's syndrome surgery, grommets, cholesteatoma surgery, hearing aid implant (magnet implantation for bone anchored hearing aids), midgut malrotation repair, duodenal stenosis repair, twisted colon repair, reconstruction of middle ear bones	rotation, duodenal stenosis						
5	Bladder infections, treated with antibiotics	-	Scoliosis	-	-	-	NA	Grommets, adenoidectomy	Plagiocephaly	Hypermetropia, strabismus	-	-	-	NA	-

6	-	-	-	-	-	-	Incidental cyst (cleft)	Umbilical & epigastric hernias	-	Strabismus	Asymmetrical	Mixed	Mild	Mod, 55 Bayley cognitive score	-
7	-	-	Orthotics for ankles & knees	Abnormal ECG	-	-	Unspecified	Dental, eyes, hernia	Aspiration on thin liquids	Hypermetropia	Asymmetrical	Sensorineural	Mod	Mod	-
8	↑ amniotic fluid, abnormal ultrasound results	-	-	-	-	-	NA	Eyes, gastric constriction	-	Hypermetropia, Squint	-	-	-	Mod	NA
9	Mother epilepsy & on medication	Seizures, failure to thrive, breech, emergency caesarean	-	-	PCOS	-	-	Grommets, adenoidectomy, tonsillectomy, G/PE tube, fundoplication	Anxiety disorder, OCD	Strabismus	Symmetrical	Sensorineural	Mild	Mod, Verbal IQ 45	
10	-	Resuscitation, ↓ oxygen	Collapsed arches & tendon/ligament damage to feet & ankles	Abnormal ECG	-	-	NA	Grommets, hypospadias, cryptorchidism	-	-	-	-	-	Mod	-
11	Lack of foetal movement	Caesarean	-	-	-	-	-	-	-	-	NA	NA	NA	Mild, FSIQ 65	NA
12	↓ foetal growth between	-	-	-	-	-	NA	Umbilical hernia	-	Hypermetropia	NA	NA	NA	NA	-

	28- & 30-wks														
13	↓ amniotic fluid	Emergency caesarean	-	-	-	-	-	-	-	Hypermetropia, strabismus	-	-	-	NA	NA
14	-	Caesarean, transverse position, umbilical cord knot, resuscitation, jaundice	-	-	-	-	Periventricular leukomalacia	-	-	Hypermetropia, myopia, Squint	-	-	-	Mod	-
15	↑ amniotic fluid	Meconium staining	-	Abnormal ECG	-	Hx absence	Small frontal lobes	Grommets, inguinal hernia	-	-	Symmetrical	Conductive	Mod	Sev	NA
16	-	-	Foot splayed outwards	Bicuspid aortic valve	-	-	Enlarged Virchow-Rowe spaces	Reimplantation of ureter	-	Myopia	-	-	-	Mod, FSIQ <50	-
17	-	Foetal distress, meconium straining, initially unresponsive	Abnormal movements or tics	Very small tricuspid regurgitation due to a floppy valve	-	1 seizure	NA	Dental, umbilical	Anxiety disorder	-	-	-	-	Sev	+
18	-	Broken clavicle	Tremor	Abnormal ECG - arrhythmia	-	Grand mal	Stable area of encephalomalacia along R lateral ventricle	Heart surgery (ablation & maze)	-	-	-	-	-	Mod, FSIQ 42	-
19	Migraines, anaemia, ↑	Mother had flash pulmonary oedema,	Radial club hand	ASD, VSD	-	-	-	Adenoidectomy, tonsillectomy,	-	-	-	-	-	NA	NA

	amniotic fluid	breathing issues						hernia, G/PE tube, circumcision, pollicization, centralization on L arm, ulnarization on L arm, turbinate reduction operations						
20	-	Breech, caesarean	-	-	Small kidneys	-	-	Grommets, adenoidectomy, orchiopexy, uvulectomy, sinuplasty	47, XXY	Hypermetropia	Symmetrical	Sensorineural	Mild	Mild	-
21	Maternal graves' disease, hyperthyroidism, gestational hypertension	-	-	Abnormal ECG	-	-	-	Grommets, adenoidectomy, tonsillectomy	-	-	Asymmetrical	Mixed	Mod	NA	-
22	-	-	-	-	Uterus deformity	-	-	Adenoidectomy	-	Hypermetropia	-	-	-	NA	NA
23	↓ foetal movement,	-	-	-	-	-	Thin corpus callosum	-	-	-	-	-	-	Mod	-

	abnormal ultrasound results														
24	Hypertension	↓ oxygen	CP	-	-	-	Arachnoid cyst	Grommets	Hypoglycaemia, ↑ magnesium & ↑ bilirubin at birth	Hypermetropia, strabismus	Symmetrical	Conductive	Mild	NA	-
25	-	-	Flat feet	-	-	-	Flair sequence hyperintense lesions in WM semioval centre & corona radiata correspond to periventricular leukomalacia, sequelae of hypoxic ischemia in newborn period	Grommets, eyes	-	Strabismus	-	-	-	Mod	NA
26	-	-	-	-	-	Generalized	-	Eyes	-	Strabismus, Alternating exotropia R eye preferred	-	-	-	Sev	-
27	Nasal thickening, ↓ foetal movement, lack of foetal movemen	Jaundice, GERD	Tremor, abnormal movements or tics	PFO	-	-	NA	Hypospadias, cryptorchidism	Infantile spasms, high anal tone Botox x2 in anal sphincter	-	Asymmetrical	Mixed	Mild	NA	+

	t, ↑ amniotic fluid														
28	-	Jaundice, reflux	-	-	-	Suspect, Petite, had seizure after contracting COVID-19	Unspecified	Umbilical	Anaemia at birth, Anxiety disorder	Myopia	-	-	-	Mod	+
29	Smaller than twin sister, smaller placenta & cord	-	Abnormal movements or tics, CP, mild thoracic scoliosis (30 degrees vertebra), curled toes, feet turned in, stoop progressing	VSD	-	-	Minor abnormalities consistent with possible Hx ischemia. Follow up MRI: Limited vol loss in frontal lobes, but overall parenchymal volumes are relatively preserved	Curled toes, gall bladder surgery	Hx gastritis & oesophagitis, gastric antral diverticulum, inflamed gall bladder, Hx migraines, Type 2 diabetes	Hypermetropia	-	-	-	Mod	NA
30	↓Maternal hormone levels, no detectable foetal heartbeat	Subarachnoid haemorrhage, subdural haemorrhage, frontal lobes	Abnormal movements or tics	Hypertension	Urinary retention, undescended testicle, swollen testicles at birth	Grand mal	-	Grommets, cryptorchidism, Botox in parotid glands (Botox spread to	Toe fungus, ↑ sweating,	Hypermetropia, wandering eyes	-	-	-	Mod, FSIQ 46	NA

		were damaged from a high forceps delivery						throat caused pureed diet for 3 months), insertion of Picc Line which caused Fentanyl overdose in infancy							
31	Transverse position, ↓ foetal movement	↓ oxygen	-	-	-	-	NA	-	-	Not specified	Symmetrical	Mixed	Mild	Mod	-
32	-	-	-	-	-	-	Cerebellar vermis & pons slightly atrophic, surrounding CSF space prominent, inferior aspect of 4th ventricle open appearance. Possible mild vol loss of the lateral aspects of cerebellar hemispheres. Lateral ventricles mildly	-	↑ red blood cells in infancy	Squint	Asymmetrical	Mixed	Mild	Mod	-

							dysplastic in shape & slightly more rounded posteriorly. Patchy high T2 signal deep WM bilaterally; most prominent in peri trigonal regions & asymmetrical							
33	↓ amniotic fluid	-	Tremor, abnormal movements & tics	-	Hx enlarged L kidney	-	-	Umbilical	-	Hypermetropia, strabismus	-	-	-	Sev	-
34	↑ amniotic fluid	-	-	-	-	-	-	Umbilical	-	-	-	-	-	Mod	NA
35	-	-	DCD	Pericardial effusion	-	-	-	-	-	-	-	-	-	Sev	-
36	-	Jaundice, abdominal distention	DCD	-	-	-	-	Relieving abdominal distention in infancy	Hypothyroidism	-	-	-	-	Mild, Global score 66	-
37	-	-	-	-	-	-	↑ ventricular size & extra axial space, ↑ sulci, ↓ WM. Signs of cortical subcortical atrophy	-	-	Myopia	-	-	-	Mild	NA

38	-	-	-	-	-	-	Markedly sclerotic, thickened appearance of stapes, R>L, & R malleolar suspensory ligament, concerning for tympanosclerosis. Marked under pneumatization of mastoid air cells & middle ear cavities. Partial opacification of mastoid air cells & middle ear cavities without evidence of osseous erosion. Mild narrowing of L external auditory canal. Possible dehiscence of tympanic	Grommets, adenoidectomy	Familial adenomatous polyposis	Hypermetropia	Asymmetrical	Mixed	Mild	Mild	-

							segments of facial nerve canals.								
39	-	-	-	-	Irregular genital appearance at birth	-	-	Tonsillectomy, hernia	-	-	-	-	-	NA	-
40	-	-	-	-	-	-	NA	-	-	-	-	-	-	Mild	-
41	-	Pyloric stenosis	-	-	-	-	-	Cryptorchidism	Hypoglycaemia at birth	-	-	-	-	Mod, FSIQ 41	-
42	Abnormal ultrasound results	Required intubation x2 & blood transfusion	-	Borderline hypoplastic L heart syndrome, sub aortic tag	Small R kidney	-	NA	-	Bronchiectasis	-	-	-	-	NA	-
43	-	-	-	-	-	Absence	WM abnormalities	Cryptorchidism	-	-	-	-	-	Sev	-
44	↓ foetal movement	-	-	-	-	-	NA	-	Heliobacter pylori infection	-	-	-	-	Mod	NA
45	Abnormal ultrasound results	↓ oxygen	-	Pulmonary stenosis	-	-	-	Eyes, G/PE tube	Hypoglycaemia at birth	Hypermetropia, Strabismus	-	-	-	Av, Verbal IQ 79	-
46	-	Velamentous insertion, double turn of umbilical cord	-	Abnormal ECG, Cardiac malformation	-	-	Heart MRI: altered repolarization with effort	Grommets, ear drum repair	Heliobacter pylori infection	-	-	-	-	Mod	-

		around neck, rupture of the placenta													
47	-	Quick labour, no crying, cone-shaped head	Abnormal movements or tics, ataxic movements, mild scoliosis, feet fallen arches, hypermobile, ↓ core temperature, does not sweat or have tears, poor temperature regulation	-	kidney reflux, R kidney bigger than L	Absence, gelastic seizures	-	Grommets, dental, Cryptorchidism	Very high pain threshold	Myopia, strabismus	Symmetrical	Sensorineural	Mod	Mild	NA
48	-	Placental hernia	-	Abnormal ECG	-	-	NA	Hernia, eyes, septoplasty, elbow repair after accident	Candida, hypothyroidism	Strabismus	Asymmetrical	Mixed	Mild	Av	NA

49	-	-	-	-	-	-	-	Appendicitis	-	Hypermetropia	Asymmetrical	Conductive	Mod	-	-
50	-	-	-	-	-	-	NA	Grommets, tonsillectomy	-	-	-	-	-	Av, FSIQ 83	-
51	-	Umbilical cord around neck, reflux	DCD	-	-	1 seizure	Delayed myelination	Tongue tie	Hypothyroidism, Bipolar disorder	-	-	-	-	Mod	-
52	-	Caesarean, pelvic positioning	DCD	ASD, pulmonary stenosis	-	-	-	Heart surgery	-	-	-	-	-	Mild, FSIQ 57	-
53	↓ foetal movement, ↑ amniotic fluid	Foetal distress, due to epidural anaesthesia, ↓ blood pressure, jaundice, emergency caesarean	Tremor, abnormal movements & tics	PFO	-	Partial focal	Arachnoid cyst along L posterior fossa	Adenoidectomy, ear drum repair	Cyclical vomiting syndrome, OCD	Hypermetropia, nystagmus	Asymmetrical, progressive	Mixed	Profound	Sev	+
54	Abnormal ultrasound results, gestational diabetes	Ventricular septal defect	DCD	-	-	-	-	-	OCD	Hypermetropia	-	-	-	Mod	+
55	↑ amniotic fluid, abnormal ultrasound results	-	-	-	-	-	NA	Dental	-	-	Asymmetrical	Sensorineural	Mild	Mod	-

56	?	?	-	-	-	-	NA	Endoscope	Eosinophilic esophagitis	-	NA	NA	NA	Mod	-
57	Hydronephrosis, ↑ amniotic fluid	Induced, breech	-	-	Hydronephrosis	-	NA	Grommets	Hypothyroidism	Hypermetropia, squint	Symmetrical	Mixed	Mod	Mod	-
58	-	Caesarean, breech	Postaxial polydactyly hands & 1 foot	-	-	-	-	- Operation for hands & foot	-	Myopia, CVI	-	-	-	Mod, FSIQ 55	-
59	Nasal bone poorly visible, ↑ nuchal fold thickness	-	-	-	-	-	Hypoxic damage signs	Grommets, adenoidectomy	-	Hypermetropia, strabismus	Asymmetrical	Conductive	Mild	Mild	-
60	-	-	-	-	-	-	Periventricular leukomalacia	-	-	-	-	-	-	Mild	-
61	-	-	Scoliosis	-	-	-	-	Umbilical, scoliosis	-	-	?	?	Mod	Mild	NA
62	↓ foetal movement, ↑ amniotic fluid, abnormal ultrasound results, late percutaneous transthoracic needle biopsy	Transient tachypnoea of the newborn, patent ductus arteriosus, jaundice	DCD, congenital crooked feet	-	-	-	Microcephaly	Inguinal, umbilical, cryptorchidism, anti-reflux valve gastrostomy, Ponseti method treatment	Moderate dysphagia	Nystagmus	-	-	-	NA	-

63	↑ amniotic fluid	-	-	Cardiac malformation	-	-	-	Removal of toe growth	-	-	?	?	?	?	-
64	Pre-term labour 25 wks, pulmonary stenosis diagnosed in utero, ↓ foetal movement, ↑ amniotic fluid, abnormal ultrasound results	Long labour, vacuum extraction	DCD, tremors, abnormal movements or tics	Pulmonary stenosis, coarctation of aorta & high-grade atrioventricular block	-	-	Slowing in front parietal lobe & gliosis in WM	Adenoidectomy, tonsillectomy, heart monitoring device implanted age 12 for atrioventricular block	Hypothyroidism, Alagille syndrome	Myopia, Strabismus, amblyopia, convergence insufficiency	Asymmetrical	Mixed	Mild	Mild, FSIQ 64	+
65	-	-	-	-	-	Grand mal	-	Umbilical	-	Hypermetropia	Symmetrical	Sensorineural	Mod	Mod	NA
66	-	Maternal 3rd degree tear	-	-	-	-	Small arachnoid cyst of the L middle cranial fossa.	Dental	-	Hypermetropia, nystagmus	-	-	-	NA	-
67	-	Small hole in heart, self-repaired	-	-	-	-	-	-	-	Hypermetropia	-	-	-	Av, FSIQ 87	-
68	-	Heart rate dropped, emergency caesarean	-	-	-	-	↓ size & intensity of supratentorial T2 WM hyperintensities	Grommets, adenoidectomy	-	Hypermetropia, strabismus	Asymmetrical	Sensorineural	Mod	Sev	-
69	-	Required breathing assistance	-	-	-	-	-	-	-	-	-	-	-	NA	+

70	-	Forceps delivery, breathing assistance, hole in heart & mild pulmonary stenosis, hemangioma on R eyelid	-	ASD, pulmonary stenosis	-	-	-	Grommets, adenoidectomy, tonsillectomy	-	Myopia, astigmatism	Asymmetrical	Mixed	Mod	Av, FSIQ 90	-
71	-	-	-	ASD, VSD, pulmonary stenosis	-	-	-	Grommets, ear drum repair	-	-	Symmetrical	Conductive	Mild	Mild, FSIQ 56	-
72	-	Vacuum assisted birth	-	-	-	-	-	Ear drum repair	-	Myopia	Symmetrical	Conductive	Mod	Av, FSIQ 85	-
73	-	Cyanosis, tachycardia	-	-	-	-	-	Grommets	Anxiety disorder, social communication disorder	Convergence insufficiency	-	-	-	Av	-
74	-	-	-	-	-	-	NA	-	-	-	-	-	-	Av	-
75	-	-	-	-	-	-	NA	Grommets	-	Hypermetropia	-	-	-	Mod	-
76	↓ foetal movement	Induced, foetal distress, emergency caesarean, breathing difficulties,	Spasticy	-	-	-	MRI hyperintense images	Grommets, adenoidectomy	-	Hypermetropia, nystagmus	Asymmetrical	Conductive	Mod	Av, FSIQ 95	-

		inconclusive newborn hearing screening													
77	-	-	Ataxic movements	PFO	-	Absence	Brachycephalic. Corpus callosum hypoplasia. Empty sella appearance. Mild prominence of the optic nerve sheath complex. here are multiple linear & punctate foci of FLAIR hyperintensity scattered throughout the subcortical & periventricular WM of frontoparietal lobes. Confluent area of T2 prolongation within posterior periventricular WM. Mild	Grommets, tonsillectomy, G/PE tube	Laryngeal cL	Myopia, squint	Asymmetrical	Sensorineural	Mod	Mod	-

							asymmetry of lateral ventricles likely representing normal variation. Visualized paranasal sinuses demonstrate mild-mod mucosal thickening								
78	?	?	-	-	-	-	Discrete alteration of morphology of cortical sulci & gyri in frontal operculum	-	-	Hypermetropia, squint	-	-	-	Mod	NA
79	-	↓ APGAR, umbilical cord wrapped around wrist	-	Pericardial agenesis	-	-	-	Umbilical	Ulcerative colitis	-	Symmetrical	Conductive	Mild	Mod, FSIQ 50	-
80	-	1200mL blood loss during childbirth, foetal tachyarrhythmia, high heart rate	-	-	-	-	Intraventricular cysts	Grommets, adenoidectomy	-	-	-	-	-	NA	NA

81	-	-	-	-	-	-	-	-	-	Hypermetropia, squint	-	-	-	Mod	-
82	-	Induced, suction cup delivery, jaundice, dusky episodes	DCD	Pulmonary stenosis	-	-	Hypoplasia of corpus callosum	Grommets, adenoidectomy, Toupet fundoplication	Chronic reflux	Unspecified	-	-	-	Mod	-
83	-	Induced, suction cup delivery, membrane rupture, heart rate ↓, neonatal infection, small hole in heart self-repaired	-	-	-	-	NA	Hydrocele	-	-	-	-	-	Av, FSIQ 89	-
84	?	?	-	-	-	-	Malrotation of L hippocampus, cysts in choroid plexuses in atria of lateral ventricles, e largest on L	-	-	Hypermetropia, squint	-	-	-	NA	-
85	-	-	DCD	Heart murmur	Enlarged kidney	-	R lateral cerebral ventricle wider than L. L periventric	Adenoidectomy, ear drums	-	Hypermetropia, strabismus	-	-	-	Av, FSIQ 8t^{h} centile	-

							ular 2 punctiform MR signal hyperintensities seen in FLAIR sequence in sense punctiform gliosis lesions								
86	-	-	-	-	-	-	-	-	-	-	-	-	-	NA	-
87	-	Induced, long labour, emergency caesarean	-	ASD	-	-	Small craniofacial ratio. Ventriculomegaly of lateral & 3rd ventricles & prominent extra-axial spaces evident. R insular cortex slightly thicker & nodular with slightly ↓ grey-WM differentiation & mild PMG here could not be excluded	Cryptorchidism	-	Hypermetropia	-	-	-	NA	+

88	-	-	-	-	-	-	Small pituitary gland	Grommets, eyes	-	Squint	-	-	-	Mod, FSIQ 50	+
89	-	Long labour, emergency caesarean	DCD	-	-	-	NA	Grommets, appendicitis	-	Hypermetropia, Squint	-	-	-	Mild	-
90	-	Induced, long labour, emergency caesarean	-	PFO	-	-	Partially empty sella, hypoplasia of corpus callosum	Dental	-	-	-	-	-	Mild, FSIQ 67	-
91	-	-	-	-	-	-	-	Cryptorchidism	-	-	-	-	-	Mild, FSIQ 55	-
92	↑ amniotic fluid	Suck/swallow reflux	-	-	-	-	↑ WM	-	Hypoglycaemia	Hypermetropia, Myopia, Strabismus	Asymmetrical	Sensorineural	Mild	Sev	-
93	-	-	-	-	-	-	-	Cryptorchidism	-	Hypermetropia	-	-	-	Mod	-
94	-	↓ movement & baby in distress, emergency caesarean, ↓ birth weight,breathing support, sepsis	-	-	-	-	NA	Adenoidectomy	Hypoglycaemia	Hypermetropia	Asymmetrical	Conductive	Mild	NA	+
95	↓ foetal movement	Forceps delivery	DCD	-	-	-	Small linear defects of distal L transfers	Adenoidectomy	-	-	-	-	-	Mod	-

							sinus seen only conventional T1 spin echo post contrast images, linear defect inferior superior sagittal sins & vital R transverse sinus. Near complete opacification bilateral mastoid air cells which demonstrates thin peripheral enhancement. Opacification bilateral middle ear cavities. R vertebral dominant. Bilateral upper cervical lymphadenopathy, likely reactive								
96	Abnormal ultrasound results	Cardiac arrhythmia, supravent	Ataxic movements	-	-	-	NA	-	-	Nystagmus	-	-	-	NA	-

		ricular tachycardia													
97	Intermittent vaginal bleeding first 3 months of pregnancy, ↓ foetal movement	Umbilical cord around neck, foetal distress, forceps delivery, thoracic retraction with breathing, subdural hematoma, blood in L ventricle, apnoeic episodes, reflux	DCD, mild R hemiplegia	Abnormal ECG	-	-	Small arachnoid cyst within R middle cranial fossa, mega cisterna magna, subtle vol loss of superior cerebellar hemispheres	Grommets, tonsillectomy	Grave's disease onset 26 years old	Myopia, Strabismus	Symmetrical, progressive	Mixed	Sev	Mod	NA
98	-	-	-	Pulmonary stenosis, supraventricular tachycardia	-	-	-	Grommets, adenoidectomy	-	Hypermetropia	Asymmetrical	Conductive	Mod	Mod	NA
99	-	-	-	-	-	-	-	Grommets	-	Hypermetropia, Strabismus	-	-	-	Mod	-
100	↑ nuchal fold thickness, abnormal ultrasound results	-	DCD	-	-	Febrile	NA	Grommets, circumcised due to recurrent urinary tract infections	-	Myopia, Strabismus	-	-	-	Av, FSIQ 77	-

101	Abnormal ultrasound results	-	-	-	-	-	NA	Grommets	IgA deficiency	-	-	-	-	NA	-
102	↓ heart rate at 40 wks	↓ heart rate, emergency caesarean, umbilical cord around neck, ↑ vomiting due to reflux, pyloric stenosis Umbilical cord around neck x3	-	-	-	-	WM hyperintensities predominantly in parietal WM	Grommets	-	-	-	-	-	NA	-
103	-	Caesarean	-	-	-	-	-	Adenoidectomy, tonsillectomy, eyes	-	-	-	-	-	Mod	NA

^ = as assessed by caregivers using the Development and Neurobehavioural Regression Questionnaire, ↓ = decreased or low, ↑ = increased or high, + = present, - = absent, ? = present, details not specified, ASD = atrial septal defect, CP = cerebral palsy, CSF = cerebrospinal fluid, CT = computed tomography, CVI = cortical visual impairment, DCD = developmental coordination disorder, ECG = electrocardiogram, FSIQ = full scale intelligence quotient, G/PE tube = Gastrostomy/Percutaneous Endoscopic tube, Hx = history of, L = Left, MRI = magnetic resonance imaging, NA = not assessed, OCD = obsessive compulsive disorder, PCOS = polycystic ovary syndrome, PFO = patent foramen ovale, R = Right, Vol = volume, VSD = ventral septal defect, wks = weeks, WM = white matter

Hearing loss: Mild (25-39dBHL), Moderate (Mod, 40-69dBHL), Severe (Sev, 70-89dBHL), Profound (>90dBHL)

Cognitive ability: Average (Av, IQ >70), Mild (IQ 55-70), Moderate (Mod, IQ 35-55), Severe or below (Sev, IQ <35).

Supplementary Table 4 Milestones by genotype in 102 individuals with Kleefstra syndrome

	DELETIONS			POINT VARIANTS				OTHER	TOTAL COHORT‡
	>1Mb	Other†	<1Mb‡	Nonsense	Frameshift	Splice site	Missense	Balanced translocation	
N¶	7	11	34 (33 with data)	17	16	8	9	1	103 (102 with data)
FIRST WORD									
12 months	1/7, 14%	1/11, 9%	2/33, 6%	1/17, 6%	1/16, 6%	2/8, 13%	1/9, 11%	0/1, 0%	9/102, 9%
12-15 months	1/7, 14%	4/11, 36%	6/33, 18%	4/17, 24%	3/16, 19%	1/8, 13%	2/9, 22%	0/1, 0%	21/102, 21%
15-18 months	0/7, 0%	3/11, 27%	3/33, 9%	3/17, 18%	3/16, 19%	1/8, 13%	2/9, 22%	0/1, 0%	15/102, 15%
≥18 months	2/7, 29%	2/11, 18%	13/33, 39%	6/17, 35%	9/16, 56%	3/8, 38%	4/9, 44%	1/1,100%	40/102, 39%
Still learning skill	3/7, 43%	1/11,9%	9/33, 27%	3/17, 18%	0/16, 0%	1/8, 13%	0/9, 0%	0/1, 0%	17/102, 17%
FIRST SENTENCES									
2-3 years	0/7, 0%	1/11, 9%	5/33, 15%	0/17, 0%	5/16, 31%	0/8, 0%	4/9, 44%	0/1, 0%	15/102, 15%
4-5 years	2/7, 29%	3/11, 27%	10/33, 30%	8/17, 47%	6/16, 38%	4/8, 50%	2/9, 22%	0/1, 0%	35/102, 34%
6-7 years	0/7, 0%	2/11, 18%	4/33, 12%	0/17, 0%	0/16, 0%	0/8, 0%	1/9, 11%	0/1, 0%	7/102, 7%
≥8 years	0/7, 0%	1/11, 9%	0/33, 0%	2/17, 12%	0/16, 0%	0/8, 0%	1/9, 11%	0/1, 0%	4/102, 4%
Still learning skill	5/7, 7%	4/11, 36%	14/33, 42%	7/17, 41%	5/16, 31%	4/8, 50%	1/9, 11%	1/1,100%	41/102, 40%
SIT WITHOUT SUPPORT									
4-7 months	0/7, 0%	0/11, 0%	1/33, 3%	0/17, 0%	1/16, 6%	0/8, 0%	0/9, 0%	0/1, 0%	2/102, 2%
8-10 months	4/7, 57%	1/11, 9%	8/33, 24%	7/17, 41%	5/16, 31%	1/8, 13%	4/9, 44%	0/1, 0%	30/102, 29%
11-12 months	0/7, 0%	6/11, 55%	8/33, 24%	5/17, 29%	7/16, 44%	2/8, 13%	2/9, 22%	0/1, 0%	30/102, 29%
≥13 months	2/7, 29%	4/11, 36%	16/33, 49%	5/17, 29%	3/16, 19%	5/8, 63%	3/9, 33%	1/1,100%	39/102, 38%
Still learning skill	1/7, 14%	0/11, 0%	0/33, 0%	0/17, 0%	0/16, 0%	0/8, 0%	0/9, 0%	0/1, 0%	1/102, 1%
CRAWLING									

7-10 months	0/7, 0%	1/11, 9%	2/33, 6%	1/17, 6%	2/16, 13%	0/8, 0%	0/9, 0%	0/1, 0%	6/102, 6%
11-13 months	1/7, 14%	3/11, 27%	8/33, 24%	7/17, 41%	5/16, 31%	2/8, 13%	3/9, 33%	0/1, 0%	29/102, 28%
≥14 months	5/7, 71%	6/11, 55%	20/33, 61%	9/17, 53%	9/16, 56%	6/8, 75%	6/9, 67%	1/1,100%	62/102, 61%
Still learning skill	1/7, 14%	1/11, 9%	3/33, 9%	0/17, 0%	0/16, 0%	0/8, 0%	0/9, 0%	0/1, 0%	5/102, 5%
WALKING									
9-12 months	0/7, 0%	0/11, 0%	0/33, 0%	0/17, 0%	1/16, 6%	0/8, 0%	0/1, 0%	0/1, 0%	1/102, 1%
13-15 months	0/7, 0%	0/11, 0%	2/33, 6%	0/17, 0%	2/16, 13%	0/8, 0%	0/1, 0%	0/1, 0%	4/102, 4%
≥16 months	5/7, 71%	10/11, 91%	28/33, 85%	16/17, 94%	13/16, 81%	8/8, 100%	9/9, 100%	1/1,100%	90/102, 88%
Still learning skill	2/7, 29%	1/11, 9%	3/33, 9%	1/17, 6%	0/16, 0%	0/8, 0%	0/1, 0%	0/1, 0%	7/102, 7%

† = other deletions detected by fluorescence in situ hybridisation, or without specific location and size of deletion specified in chromosomal microarray report

‡ = denominators reflect how many individuals have provided data/were assessed for each area, ¶ = milestones not known for one individual