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Beyond language:
Exploring the potential for combined
linguistic and molecular measures in
Fragile X Premutation Carriers
(FXPCs).

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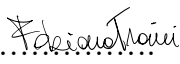
Fabiana Traini

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Summary

Biomarkers are measurable indicators of biological processes that respond to interventions and are crucial in drug development, particularly for rare disorders lacking validated measures. Particularly, complex biomarkers integrating interdisciplinary data can help identify new clinical targets. Fragile X Syndrome (FXS) and Fragile X Premutation Carriers (FXPCs) have a CGG repeat expansions on the *FMR1* gene, which encodes the FMRP protein, essential for brain function. In FXS, >200 CGG repeats cause silencing of the gene *FMR1*, and loss of the FMRP protein leading to the neurodevelopmental disorder FXS affecting 1 in 4,000 males and 1 in 5,000–8,000 females. FXPCs carry what is called a premutation (55–200 repeats) impacting 1/148 females and 1/290 males, increasing the risk of cognitive, psychiatric, and motor symptoms, including Fragile X Tremor Ataxia Syndrome (FXTAS), a Parkinson-like disorder. To date there is no cure for FXS and there is still a lack of research on the associated conditions and disorders; new measures to advance treatment discovery are therefore needed. Language abilities are promising non-invasive markers in neurodevelopmental and neurodegenerative disorders. FXS patients show expressive language deficits, while FXTAS patients exhibit disfluent speech, suggesting disruptions in common linguistic mechanisms like word retrieval. Additionally, plasma-based biomarkers are gaining ground as less invasive alternatives to CSF for detecting protein dysregulation in these conditions.

Here we combine the investigation of language abilities and plasma-based proteins to detect potential differences between FXPCs and a control group, that could point to cognitive alteration. Additionally, we retrospectively measured the same plasma-based proteins on FXS patients' samples. We used three linguistic tasks —a picture naming task, a visual word paradigm, and a picture description task — to assess language production and reception in FXPCs, with both online and in-person participants, and we collected blood samples to measure three different plasma-based proteins —acetylated α -tubulin, tyrosinated α -tubulin, and Neurofilament Light Chain (NfL) —. Our findings confirmed the presence of detectable differences between the groups:

- The FXPCs group displayed potentially delayed Reaction Times (RTs) and a more disfluent language pattern, potentially caused by disruption to the lexical retrieval process, often disrupted in neurodegeneration, as the presence of correlations among task performance revealed.
- FXPCs and FXS patients showed dysregulation of plasma-based proteins, potentially indicating similar altered mechanisms.

- Disfluencies were correlated with plasma proteins dysregulation, suggesting potential measurable connections between the biochemical markers and language characteristics.

In conclusion, this research provides a first step towards the identification of measures that combined could help advance treatment discoveries for FXS and FXPCs. In addition, it shows the importance of fuelling research through patient engagement to achieve the overall goal of improving the patient's quality of life and that of their family.

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Chapter 1: The potential for advancing biomarker discovery in Fragile X Syndrome and Fragile X Premutation Carriers.

1.1 Introduction

Fragile X Syndrome (FXS) patients and Fragile X Premutation Carriers (FXPCs) are affected by genetic mutations caused by an expansion in trinucleotide repeat CGG in the *FMR1* gene (Fragile X Messenger Ribonucleoprotein 1) [1]. FXS is a neurodevelopmental disorder characterised by an excessive CGG trinucleotide expansion, called full mutation (>200 CGG nucleotide repeat expansion) [2]. The full mutation leads to silencing of the gene resulting in the absence of FMRP, a protein crucial for typical brain development. Absence of FMRP is linked to various symptoms, including developmental delays, anxiety, and autism spectrum disorders. The prevalence rates indicate that FXS affects approximately 1 in 4,000 to 8,000 females and 1 in 5,000 males. While there is no cure, several treatment options focus on managing symptoms, such as medications for anxiety and ADHD [3]. Understanding these aspects is vital for providing support and interventions for affected individuals. While individuals with the premutation (55-200 CGG repeat expansion) were originally thought to be unaffected carriers, simply passing the mutation on to future generations, potentially leading to FXS, it recently became clear that the premutation itself can lead to several health issues [4]. These include neuropsychiatric conditions like anxiety and depression, Fragile X-associated Primary Ovarian Insufficiency (FXPOI), and a neurodegenerative disorder called Fragile X-associated Tremor Ataxia Syndrome (FXTAS), which involves tremor, cerebellar ataxia, executive dysfunction, and dementia [5]. These conditions are believed to be caused by RNA toxicity, due to the excess production of abnormal mRNA, which interferes with normal cellular function. Unfortunately, there are currently no cures or diseases-modifying therapies, only symptom management is available [6]. This makes it crucial to develop better biomarkers and therapeutic strategies to advance treatment discovery, to fulfil the several unmet medical needs for both these populations. Additionally, there are limited research on FXPCs and even the available findings sometimes are poorly understood. For instance, there is now evidence that FXPCs can display a more disfluent speech pattern compared

to controls [7], however the nature of these disfluencies is unknown, and several studies failed in identifying the origins [8]. Therefore, there is a need for new measures in this population, that are objective, reliable, reproducible and feasible. Particularly, biomarkers are needed to tailor appropriate therapies. Biomarkers are measurable indicators of processes that change in response to an intervention; they can be used as predictive measures of a diseases or as endpoint measure in clinical trials [9]. There are several types of biomarkers, but particularly complex biomarkers, made up of different measures, have shown potential to become powerful tools in treatment discovery, especially in rare disorders, where there is a need for reliable measures that could advance treatment discovery [9]. Language abilities have been often used in neurodevelopmental disorders as measurable endpoints in clinical trials [10], and in neurodegenerative disorders as early markers of cognitive alterations [11]. Expressive language disruptions have been observed in FXS patients and language dysfluencies have been reported in FXPCs, potentially indicating disruption in the lexical retrieval process [12]. Additionally, plasma-based biomarkers have become popular measures as less invasive alternatives to CSF collection, to detect proteins dysregulations to inform about the general health status of the body [13].

Here we put together language and molecular measures; we combined a trio of linguistics tasks, and we collected blood samples to measures three plasma-based proteins to determine potential differences between FXPCs and control participants (CP). Additionally, we measured the same plasma-based proteins on FXS patients. with the main aim of identifying differences between FXPCs and controls participants that could potentially indicate that these measures are valuable to detect signs of alterations before the development of FXTAS and that could potentially be used in further studies to explore new therapies pathways. In the following sections of Chapter 1, we will further explore the rationale for combining these measures, and we will first focus on describing FXS and FXPCs and associated conditions, and the available therapies. We will then focus on describing the different types of biomarkers, particularly in neurodevelopmental and in neurodegenerative disorders. We will then address the measures we chose to investigate, to potential contribute to develop a complex biomarker. We will first describe language in FXS, and the similarities identified with FXPCs, but we will then exclusively focus on language in FXPCs in the context of language

studies in neurodegeneration. We will then move on to describe the molecular aspects and the proteins of interest and how they have been investigated in similar disorders. We will finalise the first chapter with a description of the importance of Patient and public involvement (PPI) and the role played by it in this project, particularly considering the need for tailored and feasible measures for this population. The subsequent chapter will be dedicated to address the four main research questions.

Chapter 2 will be dedicated to the linguistic measures and to address the relevant research questions: a) Is it possible to identify potential differences between FXPCs and a control group through language assessment that could indicate prodromal signs of cognitive alterations in FXPCs? b) What is the nature of language disfluencies patterns in FXPCs and are these related to a primary language deficit?. We will first give an introduction on the theoretical background and rationale of the linguistic measures chosen (investigating lexical access), and we will then explore the methods, results and discussion for the first experiment.

Chapter 3 will be focused on the molecular measurements and on combining the linguistic and molecular aspects, addressing the relevant research questions: c) Is it possible to identify potential alterations in plasma-based proteins in FXPCs and in FXS? Are these alterations similar? d) Is it possible to find links between linguistic and molecular measures by combining molecular and linguistic measures to advance development of treatments for FXPCs?

Once again we will further explore the rationale and background for the molecular measures chosen in the molecular experiment and for combining the two types of measurements, and we will then explore the methods, results and discussion.

Finally, in Chapter 4 we will address the general discussion, focusing on summarising the main results first, giving our interpretation and finally what are the strengths and limitations of the study and the possible future directions.

1.2 Fragile X syndrome and Fragile X Premutation Carriers (FXPCs)

1.2.1 Background

In recent years many incentives have been developed to enhance research and drug discovery in rare diseases, and to treat it as a public health priority, but many challenges remain [14]. Among all, one of the biggest challenges is that drug development is based on a risk benefit assessment and the risks in clinical trials for rare disorders can be many, especially considering the heterogeneity of the population and the lack of research [15]. One of the main issues in drug discovery research is the identification of appropriate biomarkers and clinical endpoints, and this becomes even more challenging in rare disorders where most of the times there are no previous validated scales, clinical endpoints, and measures in general [16]. Therefore, there is a need to research new measures to accelerate this process, not to mention the necessity of the improvement in diagnostic criteria as many genetic rare disorders are often mis or underdiagnosed. Complex biomarkers, combining different measures are particularly useful for this purpose, and they may represent the best way forward in discovery, as it is difficult to find an appropriate single measure that can unambiguously be used for developing therapeutic targets [9]. One single candidate biomarker, if not matched with other measures, can lead to erroneous or inconclusive results, and ultimately to a waste of resources or risk to patients. Following this line of investigation, this study will focus on two rare disorders, namely Fragile X Syndrome (FXS) and on Fragile X-Associated Tremor Ataxia Syndrome (FXTAS), both caused by different mutations on the *FMRI* gene. Although they are two different conditions, as one is a neurodevelopmental disorder with a paediatric onset (FXS), and the other is a neurodegenerative disorder with a late onset (FXTAS), we will focus on both disorders. First, because many aspects of the impact that an *FMRI* mutation has are still largely unknown and we believe that investigating two different conditions linked to the same gene mutations, could potentially improve our knowledge of the effects of the underlying mechanisms. Secondly, because most of the time the individuals at risk of developing FXTAS, Fragile X Premutation Carriers, are also caregivers of children with Fragile X Syndrome. They often do not know what the future will hold for them, as there is still a huge gap between the many research on FXS

and the lack of knowledge on FXPCs. Consequently, the whole family is hugely impacted when a diagnosis of one or the other comes, and the only way to improve the quality of life of the whole family is to involve parents as well as children in the research process. In this project we will start by focusing on FXPCs and then we will also present results of an exploratory study on FXS samples. In the next paragraphs we will talk about the epidemiology and biology of FXS and FXPCs and then we will focus particularly on the need of advance biomarker discovery in FXPCs, the subjects of the main study of this project. Then we will further expand the above-mentioned idea of a complex biomarker that includes language and molecular analysis, and we will discuss the rationale for both kinds of assessments. The last section of this introductory chapter 1.6 will focus on the patient centric approach of this project and on the importance of Patient and Public Involvement in Research (PPI) in all stages of the research process to empower patients, to effectively investigate their needs and to reach communities around the world.

1.2.2 Fragile X Syndrome (FXS) Aetiology and epidemiology

FXS is a neurodevelopmental disorder caused by the loss of functions of the *FMRI* gene (Fragile X Messenger Ribonucleoprotein 1 gene). The *FMRI* gene is located on the X-chromosome and produces the fragile X messenger ribonucleoprotein (FMRP), whose functions are essential for typical brain development. Many of the biological functions of FMRP are still unknown but we know that is critical to development of the brain because it controls translation of specific messenger RNAs (mRNAs) that plays a fundamental role in synaptic plasticity [17]. FXS is a repeat disorder meaning that the mutation which causes loss of functions of the gene is a CGG trinucleotide repeats expansion. The development of the neurodevelopmental disorder FXS is linked to the full mutation (more than >200 CGG nucleotide repeat expansion) and methylation of the promoter which causes silencing of the gene, nonetheless many mechanisms responsible for FXS are yet to be discovered and understood. It is estimated that around the world 1 in 4000 males and 1 in 5000 to 8000 females live with FXS [3], and it is considered the leading inherited cause of intellectual disability: about 30% of females and 90% of males affected by the disorder have intellectual disability, while around 60% of males are diagnosed with Autism Spectrum Disorder (ASD) [3]. In terms of clinical presentation,

there is a high within-syndrome variability, and symptoms and phenotypical features can vary. Developmental delays affect about 90% of individuals, both males and females and around 40/60 % will be diagnosed with ASD [18]. Other common diagnosis among individuals with FXS is attention deficit hyperactivity disorder (ADHD) and anxiety, which could present differently from individual to individual across the lifespan. Further behavioural characteristics, include aggressiveness and obsessive-compulsive disorder. Additionally, around 20% of individuals can suffer from seizures, sleep disturbances and obesity[2]. Females usually display a milder phenotype due to the protective effect of the second X chromosome which usually contains a healthy copy of the *FMRI* gene, and it is estimated that more than 70% of girls have a general low IQ [3].

Males are more often affected by language impairment and delays. Language in FXS has been studied by many research groups, and we will discuss the linguistic profile of FXS further in 0.

1.2.3 Fragile X Premutation Carriers (FXPCs) biology and epidemiology

FXS is caused by a full mutation on the *FMRI* gene (>200 repeat expansions), but this is not the only kind of mutation that can occur on this gene. The normal range is <45 CGG repeats expansion, while individuals who are in the 55 to 200 range are considered premutation carriers. Individuals who carry a premutation in the *FMRI* gene are at risk of developing neuropsychological, cognitive, and motor related symptoms, including language abilities difficulties [19]. It is estimated that 1/148 females and 1/290 males are *FMRI* premutation carriers [20]. Historically and up until recently, the only risk associated to being a premutation carrier, was to have a child with the full mutation. In the early 2000 this conception was subverted by the discovery of several conditions associated to the premutation range[4]. The reason why premutation carriers might be subject to develop different conditions seems to be associated with RNA toxicity, as the elevated number of CGG repeats correspond to an elevated level of *FMRI* mRNA [6] [21]. These elevated levels can cause several issues including calcium dysregulation, oxidative stress and neuronal cell death [6]. The most studied conditions associated to premutation carriers are: Fragile X Primary Ovarian insufficiency (FXPOI) which affects about 20-28 % of females, a Parkinson-like neurodegenerative disorder, Fragile X Tremor Ataxia Syndrome (FXTAS), which affects about 45% of males and 16 % of females [22], and a series of

neuropsychiatric conditions regrouped under the umbrella term FXAND, Fragile X-Associated neuropsychiatric disorders, proposed in 2018 to include all the associated neuropsychiatric consequences connected to the Fragile X Premutation [23]. Although the term has been proposed to put together all the physical and neuropsychiatric conditions associated with the premutation, the use of FXAND has been challenged by the European Fragile X Network (EFXN) during a symposium held in The Netherlands in 2019, where 17 families from 16 countries convened to debate about the latest research discoveries on FXPCs [24]. During the meeting many individuals affected by the premutation expressed their concerns in being associated with the term disorder, as they will not necessarily develop a disorder. Additionally, they felt stigmatised when being labelled as having a disorder, and that the term included too many conditions which differ greatly from one another and therefore should not be put under the same umbrella term. As a result, they suggest replacing the term FXAND with Fragile X Premutation Associated Conditions (FXPAC) as an umbrella term to include both FXTAS and FXPOI. They additionally suggested to use FXANC, Fragile X Associated Neuropsychiatric conditions and FXVAC, Fragile X Various associated conditions [24] to include all those conditions who are not of a neuropsychiatric nature. However, to the best of my knowledge, these two terms have not been frequently used by the literature published ever since. The debate around the appropriate terms to be used seems to be still open and a compromise appears to be the trend of using FXPAC as an umbrella term to refer to all the associated non neuropsychiatric conditions, and FXAND to refer to the neuropsychiatric disorders [4]. Due to the importance of using the appropriate term for the right definition, as stated at the beginning of the chapter, we will use both FXPAC and FXAND in this manuscript. We will discuss more about the pathophysiology of FXPCs in chapter 2 and 3, we will focus now on the different symptoms of FXPAC and FXAND and the available treatments. The several different conditions individuals with Fragile X Premutation are exposed to can be various. They can have fibromyalgia, peripheral neuropathy, autonomic dysfunction etc, while in terms of neuropsychiatric conditions the most common are depression and anxiety, ADHD, chronic fatigue, chronic pain, and executive dysfunctions [25]. It is estimated that individuals with the premutation have 20% more risk of developing anxiety and depression compared to the general population [26], and this percentage can increase with age [27]. Additionally

recent studies found a curvilinear correlation with the number of CGG repeats, particularly those in the range of 70 to 100 CGG repeats are more affected, and if women develop FXTAS, their neuropsychiatric symptoms develop at a much higher rate [6]. ADHD is also very common, especially at a younger age, however it can persist through adulthood and worsen with aging, additionally a small percentage of individuals can be diagnosed with ASD and they can display language deficits such as dysfluencies and difficulty in word finding abilities [28]. Another common condition which affects approximately 20-30% of women with the premutation is FXPOI [29]. This condition was discovered in 1991 and can lead to early menopause (before the age of 40), or absent or irregular menstruation [30]. Once again, there seem to be a curvilinear correlation with the number of repeats, particularly the symptoms are more acute in the range of 80-100 CGG repeats [6]. The most recently discovered condition associated with the premutation is FXTAS which was discovered in 2001 by Randi Hagerman and her colleagues at the MIND Institute at UC Davis in California. Hagerman is considered one of the main experts of FXS and during routine visits of children that were brought to her clinic, she began to notice that often parents and caregivers would share similar stories about conditions that run in the family, including grandparents or family members often diagnosed with Alzheimer's Disease (AD), Parkinson's Disease (PD), various ataxia, tremors and other neurological conditions [31]. After MRI research studies correlated with patients' family histories, a correlation was found between the occurrence of a neurodegenerative diagnosis in those families and the presence of the *FMRI* premutation, which led in 2003 Hagerman and her team to propose the term FXTAS to define the neurodegenerative conditions affecting many of the family members [32]. To date, despite the efforts of many researchers and different communities, there is still a lack of research on this condition and one of the biggest challenges, is still the correct diagnosis as many patients keep being diagnosed with either PD or AD instead of FXTAS. It is only recently that research is focusing on FXPCs, therefore studies are still ongoing to understand the extent of the impact of living with a premutation of the *FMRI* gene, and there is a need to further advance biomarker and drug discovery in all the associated conditions. In the next paragraph we will discuss more in detail some of the above-mentioned conditions and the current available treatments options for FXPCs.

1.2.4 Fragile X Premutation Carriers: A population in need of more research and therapies

FXS, FXPAC and FXAND are not the same thing, but they share a common aetiology: a repeat expansion mutation on the *FMRI* gene. There is currently no cure or approved treatment for FXS or the associated conditions, and treating the conditions associated with the premutation is not easy because many different issues coexist within the same individuals, and for many of them only treatment of symptoms is possible. In a review of potential treatment options for carriers in 2021, Sodhi and Hagerman give an overview of the available treatment options for each of the three main associated conditions as presented by Sodhi and Hagerman: FXAND and FXPAC, FXPOI and FXTAS [6].

- ***FXAND and FXPAC:*** some of the most common symptoms, anxiety and depression and ADHD, are usually treated with a combination of psychotherapy and medications such as serotonin reuptake inhibitors (SSRIs) and norepinephrine reuptake inhibitors SNRIs), especially when neuropsychiatric symptoms connected to FXAND are present [6, 33].
- ***FXPOI: symptoms*** include early menopause or period absence, and ovarian insufficiency which are usually managed with Hormone replacement Therapy (HRT) Wheeler, 2014 #1010}.
- ***FXTAS:*** several different condition-related symptoms are compatible with a FXTAS diagnosis. Tremor and Ataxia are usually treated with medications for similar symptoms in PD, like propranol and other beta blockers or Sinemet for tremors at rest. Memory and cognitive issues are usually treated with medications for dementia, such as donepezil, or memantine, while peripheral neuropathy is treated with medications like topical cannabidiol or Lidocaine [6] (Table 1).

Conditions	Cause	Symptoms and diagnostic criteria	Prevalence	Treatment options
FXS	>200 CGG repeats (full mutation)	Special phenotypic features ASD ADHD Anxiety Developmental delays Compulsive disorders Seizures Sleep disturbances GI reflux Language delays	1 in 4000 to 8000 females 1 in 5000 males	No cure/only symptoms management Metformin is being studied for social behavioural problems Sertraline for depression and anxiety Minocycline considered beneficial for regulating synaptic activity Acamprosate for anxiety Lovastatin studied for improvement of sensory and cognitive functions ADHD treatments
FXAND/FXPAC	55-200 CGG repeats (premutation)	Anxiety, depression, attention deficit hyperactivity disorder (ADHD), chronic fatigue, sleep problems, behavioural changes.	70 % of Premutation carriers develop at least one anxiety disorder and earlier than controls, and women are more at risk. 15% of men develop ASD and 50 % of women.	No cure/ only symptoms management -Serotonin Reuptake Inhibitors (SSRIs) Sertraline, Fluvoxamine, paroxetine, escitalopram and fluoxetine. -Norepinephrine reuptake inhibitors (SNRIs) Duloxetine or venlafaxine for pain and depression -ADHD stimulants or non-stimulants treatments
FXTAS	55-200 CGG repeats (premutation)	Tremor, cerebellar gait ataxia, peripheral neuropathy parkinsonism, executive dysfunction and dementia, executive dysfunction. Brain white matter changes and brain atrophy.	40% of males > 50 years of age 8%-16% of females >40 years of age	No cure Hormone Replacement Therapies with oestrogens
FXPOI	55-200 CGG repeats (premutation)	Early menopause (before 40yrs of age)	20% of women with Premutation. Correlates with number of repeats, maximum in the mid- range (80-100 repeats).	No cure Drugs for movement disorders e.g. (propranolol) or drugs for PD. Drugs for cognitive dysfunctions drugs of MCI, only trial NMDA. Drugs for psychiatric issues SSRIs (Citicoline trial) Drugs for neuropathic pain CBD or Lidocaine patches

Table 1: FXS and FXS-associated disorders characteristics.

The table summarises characteristics of the different conditions associated with the FMR1 mutations and available treatments.

Considering the vast range of symptoms and the lack of specific treatments, individual targeted therapies are often recommended to ensure the best care options for each patient [34]. Additionally, there is a necessity for more clinical trials with the aim of developing new medications and interventions. Clinical trials have usually been exclusively focusing on FXS, and several options have been explored in the past decades. In this section we will not go into details about all the trials performed so far on FXS as we will focus mainly on FXPCs, but we believe it is worth mentioning that many efforts are being done to advance treatment discovery in this field. Encouragingly two clinical trials show promise and are currently recruiting participants:

- The EXPERIENCE (Evaluation of Fragile X Experience in Cognition Expression) trial managed by Shionogi Inc., that recently reached phase 2b/3 and is based on the use of a phosphodiesterase (PDE) inhibitor which showed very promising results in the previous phase on a broad range of outcome measures, including, behaviour, cognition, and electrophysiology measures.
- The RECONNECT clinical trial, managed by Harmony Biosciences which reached phase 3 and is based on the use of ZYN002, a pharmaceutically manufactured CBD, in the form of a gel to improve behavioural symptoms. ⁱ

Clinical trial	Rationale	Status	Sponsors and Collaborators
Zatolmilast, an Investigational Treatment for FXS	Efficacy of PDE inhibitor to improve behaviour, cognition and intellectual disability	Phase 2b/3 enrolling	FRAXA Tetra Therapeutics Shionogi Inc.
Clinical (RECONNECT) trial of ZYN002	Efficacy of a pharmaceutically manufactured CBD, ZYN002, in the form of a gel to improve behaviour.	Phase 3 enrolling	Harmony Biosciences

Table 2: FXS ongoing clinical trials.

This table offers an overview of the ongoing FXS clinical trials that reached phase 2b/3 and that are currently recruiting participants.

ⁱ More details can be found on the FRAXA research foundation website <https://www.fraxa.org/tetra-therapeutics-phase-3-clinical-trial-of-zatolmilast-in-fragile-x-syndrome/>.

1.2.5 Treatment options for FXPCs.

As mentioned in 1.2.3, there is a need for more research to find specific treatments for the unique situation of premutation carriers, because often medications used for other conditions are not enough to address the many heterogeneous symptoms and they need specific and targeted interventions. A few clinical trials have been held in the last decade for FXTAS, studying and testing different treatment options for the condition. An open label clinical trial, that started in 2015 and lasted until 2017 reaching Phase 2, was performed at Rush University Medical Centre to test Citicoline to treat tremor and balance and to stabilise cognitive decline. Patients were tested at 3,6, and 12 months for several cognitive and motor measures. They showed significant improvement in the Beck Anxiety Inventory and the Stroop Colour-Word test while all the other measures remained stable; safety was also tested and only a minor adverse event was registered in one patient. Therefore, citicoline seems to be safe and well tolerated, as well as resulting in promising outcomes, however further studies are needed to confirm this [35]. The NMDA antagonist memantine was also tested in a Double-Blind Placebo-Controlled Trial, at UC Davis in California, in a study completed in 2012. The results showed improvement in attention and working memory measured with Event related Potential (ERP)ⁱⁱ after only one year [36]. Some dietary supplements were also recently tested to look for potential biomarkers rather than testing clinical efficacy, like Sulforaphane, an antioxidant found in some kind of vegetables. The antioxidant was tested in an Open Labelled Trial, which ended in 2023 to study effects of this supplement on certain molecular blood biomarkers. Results showed safety and tolerability of the supplement with potential beneficial effects on cognitive functions [37]. Lastly, an open labelled clinical trial ended in 2017, where patients were treated with Allopregnanolone which is a neurosteroid and a GABA_A receptor and can promote regeneration and improve clinical symptoms. Six patients received allopregnanolone intravenous once a week for 3 months and results showed potential for improving cognitive and executive functions, learning and for alleviating some neurodegenerative symptoms measure with ERP and MRI [38].

ⁱⁱ An Event Related Potential (ERP) is an electrophysiological response to a stimulus. It is often used in language studies to measure brain response to a presented visual or auditory language stimulus.

Clinical trials	Rationale	Status	Sponsors and Collaborators
Diaphragmatic Breathing and Heart Rate Variability Training for Improving Hypertension in Fragile X Associated Tremor/Ataxia	Efficacy of a biofeedback treatment to support self-regulatory processes for heart rate and breathing patterns.	2019-completed	University of California, Davis
Open-Label Trial of Sulforaphane in Premutation Carriers With FXTAS	Testing effects of dietary supplement Sulforaphane on biomolecular markers in blood.	2023-completed	University of California, Davis
Treatment of Fragile-X Associated Tremor/Ataxia Syndrome (FXTAS) With Allopregnanolone	Testing efficacy and safety of Allopregnanolone as a treatment for FXTAS.	2017-Phase 2 2023	University of California, Davis PureTech Health awarded 11.4 million to advance oral allopregnanolone
Memantine Treatment in Fragile X-Associated Tremor/Ataxia Syndrome	Efficacy of memantine to treat FXTAS symptoms	2012-completed	University of California, Davis Forest Laboratories National Institute on Aging
Citicoline for Treatment in Fragile X-associated Tremor/Ataxia Syndrome	Efficacy of citicoline to stabilise balance abnormalities and cognitive decline.	2017-completed	Rush University Medical Centre

Table 3: FXTAS clinical trials.

This table offers an overview of the main clinical trials for FXTAS held in the past decade. Source <https://clinicaltrials.gov/>

In conclusion, the scarcity of available drugs targeted for FXTAS and in general for premutation carriers, show not only the need of more trials to find effective treatments, but also the need for more effective biomarkers to target interventions. In the next sections we will develop the idea of the importance of looking at different measures to improve clinical diagnosis and future treatment options.

1.3 What is a biomarker?

“What's in a name? That which we call a rose by any other name would smell just as sweet.”

William Shakespeare uses this quote in his play *Romeo and Juliet* to express the idea that names are just meaningless labels that we assign to things or people. This may be true in Shakespeare's tragic play, but how important are these labels in shaping our thoughts and fuelling are actions in real life? Assigning labels and definitions to spoken or written symbols is part of what defines a language and when the definition of that symbol is clear and accepted by the broad community, it helps conveying the meaning of a word and use it properly in the shared daily

endeavour to communicate effectively. This is important in every conventional system that we encounter in our day-to-day life, particularly in research, and it is also fundamental in life sciences when the centre, the main subject of the investigation, are people. The importance of giving clear and understandable definitions has been embraced by many fields exploring human biology and nature, and it takes a fundamental meaning especially when it comes to the drug discovery field. One of the biggest challenges in the field, is to identify biomarkers to target specific interventions and therapies. Biomarkers are fundamental in many aspects of research, and observational biomarkers studies are crucial for enhancing and simplifying the drug discovery process. To be more specific, appropriate identification of a biomarker could help reduce risks in clinical trials as it is focused on the drug design stage rather than the optimisation on the final stages of clinical trials, because they can have sort of a predictive power in the selection of the right drug to use[39]. In a nutshell, it provides a measurable target that can provide an idea of whether the drug is going to work or not on the specific target. Additionally, more biomarkers studies can improve the drug development process in terms of risks and safety and in terms of reducing the overall cost of drug development, as it can be helpful in selecting among different targets based on effective measurable changes, and selecting the correct measure, can ease the overall use in clinical settings [15]. Although biomarkers are an essential instrument in the drug development and medical research, sometimes definitions and terminology are not clear and can lead to confusion. A biomarker can be defined as a *“characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or an intervention”*[9]. This is quite a broad definition, but it can be applied to several different fields. A biomarker should not be confused with a Clinical Outcome assessment (COA) which measures the basic feeling, functions and surviving strategies of a person [9]. Both Biomarkers and COA can be used as endpoints in clinical trials, meaning that they can be used as a measurable variable to address a specific research question, and a measure that can be used as a biomarker could also become an endpoint measure in clinical trials. In 2018 Califf et al. [9] examines the definition of several kind of biomarkers reviewed by the U.S Food and Drug Administration and the National Institutes of Health in their joint Biomarkers, EndpointS, and other tools (BEST). Why the need for a definition, one might ask. Califf et al. argue that if we look

back at the past decades, progress in technology led the medical field into groundbreaking discoveries and the number of possible biological measurements has increased exponentially. All of this could really help in moving forward important scientific discoveries, but at the same time the overwhelming number of models, and potential measurements, can lead to confusion and a waste of resources. A clear and precise definition can help focusing since an early stage in the development of the correct and appropriate measurement, which can meet the criteria for regulations and validation and in turn become a target in clinical trials [9]. Identifying the correct target is fundamental to choose correct therapies and interventions, while misunderstood concepts or ineffective measures, can lead to a misuse of funding in development of biomarkers, and ultimately in a waste of resources that will not be used to address the main goal of drug discovery: finding therapies that can improve the quality of life of patients living with a specific condition [9]. We will now give a short definition of each biomarker described in BEST, but we will only further explore the ones that best describes the kind of measures that we aim to explore with this project. Table 1 offers an overview of the different definitions proposed by BEST.

Biomarkers	Definitions in BEST
Diagnostic biomarkers	Detect or confirm the presence of a disease or condition, or to define a specific subgroup.
Monitoring biomarkers	Monitor the progress of a specific conditions.
Pharmacodynamic/response biomarkers	Measures the efficacy of a specific treatment.
Predictive biomarkers	Predict response to a specific treatment.
Prognostic biomarkers	Measures the likelihood of the progression of the disease.
Safety	Indicate the likelihood of developing a specific disease.
Susceptibility/Risk	Likelihood of the progression of the disease.
Surrogates	Substitute for clinical outcomes and can be used to draw inferences for clinical benefits of a treatment.
Complex Biomarkers	Composite biomarkers that put together different biological systems that play a role in the clinical outcome.
Digital biomarkers	Sensors and personal devices that makes possible to and make clinical inferences on the level of cognitive abilities, motor patterns, eating and exercise habits etc. of a person.

Table 4: Summary of biomarkers definition.

This table summarises the main definitions discussed regarding the different types of biomarkers identified by BEST as described in Califf et al. [9].

Each different type of biomarker could be important to target a specific aspect of a condition, and the same measure can be used for more than one category. Depending on the challenges that a specific disease can present, one aspect can

become more important than another. For example, in neurodevelopmental disorders, surrogates or monitoring biomarkers could be particularly important to monitor disease progression as well as measuring changes in clinical outcomes [40]. At the same time, predictive and pharmacodynamic biomarkers could be fundamental when it comes to predict or measure response to a specific treatment [41]. Additionally, prognostic biomarkers could be crucial in differential diagnosis as they deal with identifying different disease outcomes, and this can become particularly important at the earliest stages of neurodegenerative disorders for instance [42]. While digital biomarkers are potentially the future in terms of applying technological discoveries to the medical field [43], complex biomarkers could represent a fundamental turning point in the drug discovery field [44], as more and more evidence suggests that one measure is not enough to identify reliable and strong changes in a condition, therefore combining different kind of measures could be the best way forward into detecting potential good candidate to be validate as biomarkers. We will further explore the idea of complex biomarkers in section 1.3. of this chapter.

1.3.1 Biomarkers in neurodevelopmental disorders

What makes a biomarker a good candidate? Any stakeholder working on biomarker and drug discovery tries to answer this question. However, there is no easy answer for it, and many different aspects should be considered. Two main features seem to be fundamental to identify a good candidate:

- reliability, which not only incorporates the quality of the science behind the discovery of a certain measure but also refers to how dependable a measure is as an accurate indicator of the concept of interest.
- reproducibility, which refers to the consistency of a measure across different studies and populations, and addresses the need for similar protocols and approaches in the different teams working on similar subjects around the world, in a word, the need for collaboration [9].

Consequently, one of the main points to consider is the different kind of approaches that have been taken in the different fields, to investigate potential good measures across similar disorders. Therefore, in this section we will summarise the main findings across some of the currently most researched neurodevelopmental disorders in biomarker discovery, focusing on the types of biomarkers that are common across disorders. Biomarkers are critical to advance

discovery of therapeutical targets in many medical fields, especially in the neurodevelopment field. In the last three decades advances in genetics and neurobiology helped in the identification of several genes responsible for neurodevelopmental disorders, including some genes that play a role in one of the most common neurodevelopmental disorders, such as autism spectrum disorder (ASD). The advancement in genetics had another important consequence, as it put at the centre of the debate the study of rare genetic diseases, particularly in the biomarker discovery field. The renewed attention to rare disorders is mainly due to three main reasons:

- a. **Known etiology:** often a gene or genes responsible for the specific disorder are well known and this can be an asset in directing research to specific therapeutical targets.
- b. **Competitive collaboration:** the heterogeneity of the rare diseases discovered so far, led to a positive competition to discover many different small molecules, measures or therapies targeting each different disease, but these discoveries and approaches can potentially advance research across other rare disorders [16].
- c. **Strength in numbers:** the parent of a child with CDKL5 Deficiency Disorder (CDD) once said, “*As small as we are, there is strength in those small numbers.*” The number of rare disease identified is between 6000-8000, whose 80% have a genetic origin and around 50/57% start at a pediatric age [45]. It is estimated that collectively, around 263 to 446 million people around the world are affected by rare diseases and if we consider the impact on caregivers and family members the number is even bigger and can reach 1.4 billion people [14, 46]. This means that despite the heterogeneity and individual peculiarities of each disease, there is a global effort to tackle common challenges and similar common misconceptions.

Therefore the consensus across biomedical research fields is that collaboratively addressing rare diseases’ common issues should be seen as a global public health priority not only to improve the quality of life of all those affected, but also to lead the way to develop similar approaches to tackle other common diseases [45]. We summarise below in Table 5 important discoveries in biomarkers research, and we focus on some representative neurodevelopmental disorders to illustrate important similarities across them.

Disorders	Cause	Symptoms	Candidate biomarkers	Type of biomarker	Prevalence
ASD (Autism Spectrum Disorder)	Highly heritable but aetiology not clear. Caused by abnormal neural development	Impaired social interactions, communication and restrictive, repetitive, behaviours	-biochemical and clinical (serotonin, melatonin excretion, head circumference, cognitive and behavioural measures) -EEG and eye tracking for auditory processes and eye movements. -TMS as a diagnostic biomarker	-fluid biomarkers (blood, plasma, urine) -EEG -TMS	1 in 100
Rett Syndrome	Mutations gene MECP2	Delays in development, regression in language and hand use, stereotypies, social withdrawal, seizures	-plasma metabolite related to oxidative stress -omics for monitor and target efficacy -EEG theta and Delta abnormalities	-fluid biomarkers (blood, plasma, urine) -EEG	1 in 10.000 females
TSC (Tuberous sclerosis complex)	Mutations gene TSC1 or TSC2	Seizures, cognitive difficulties, behaviour problems, ASD, skin abnormalities, lung lesions, kidney problems	-brain imaging for volume of specific regions -EEG auditory response to speech	-brain imaging -fluid biomarkers (blood, plasma, urine) -EEG	1 in 6.000
FXS (Fragile X Syndrome)	Mutations gene FMR1	Intellectual disability, social anxiety, hyperactivity, seizures, repetitive behaviour executive functions issues and language delays	-plasma biomarkers -EEG for auditory abnormalities -ELS (expressive language samples)	-fluid biomarkers (blood, plasma, urine) -EEG -Language sample	1 in 7.000 males and 1 in 11.000 females
Angelman Syndrome	Mutations gene UBE3A	Delayed development, intellectual disability, severe speech impairment, ataxia, epilepsy	-EEG slow wave pattern, increased Delta power and dynamics	-EEG	1 in 12.000 to 1 in 20.000
CDD (CDKL5 Deficiency Disorder)	Mutations gene CDKL5	Early onset seizures, motor cognitive and visual impairment	- plasma biomarkers - EEG/EP visual and auditory abnormalities	-fluid biomarkers (blood, plasma, urine) -EEG	1 in 40.000 and 1 in 60.000

Table 5: Biomarkers in neurodevelopmental disorders.

This table summarises findings in neurodevelopmental disorders biomarker discovery. Information based on Sahin et al. review [40]. CDD information based on Gennaccaro et al. [47] and Saby et al. [48].

Table 5 shows an overview of some rare neurodevelopmental disorder's characteristics and findings in biomarker discovery. Despite heterogeneity in aetiology, causes and symptoms the approaches in biomarkers' findings are similar:

1. **Electrophysiology** is a common denominator across neurodevelopmental disorder biomarkers studies, because it provides a minimally invasive approach for functional brain imaging, especially when dealing with children. Particularly, electroencephalographic measurements (EEG) can be used to directly investigate several brain mechanisms and patterns, and recent evidence suggests that several neurodevelopmental disorders have a signature EEG pattern that could be potentially used as a candidate biomarker [49]. EEG is an extensively used procedure that records electrical brain activity from the scalp using electrodes. It is an important technique applicable to patients of all ages without restrictions [50]. Thanks to advancement in technology new EEG wireless caps are entering the market in a way that could potentially be revolutionary for this field, as detection of EEG signal could be done remotely, at home in a less stressful way [51].

2 **Peripheral fluids**, such as blood and urine, show promise as collection of these fluids is considered to be a minor invasive procedure. In recent years evidence suggests that many different proteins could be detected in plasma as well as in cerebrospinal fluid (CSF), whose collection procedure is often too invasive for children especially [52] [53]. Peripheral plasma biomarkers have recently been investigated at Ulysses Neuroscience Ltd., the company sponsoring this PhD project, in Fragile X Syndrome (FXS) and CDD. The team was successful in detecting plasma-based biomarkers of dysregulation in both disorders that shows a correlation with similar findings in brain tissue of animal models of the diseases, suggesting a potential correlation between what has been observed in plasma and what is happening in the brain. These promising results will be further discussed in 1.5.

3. Lastly, **language** is also considered a valid measure to be used both as a candidate biomarker of disease progression, and as a clinical trial endpoint measure. The most used assessment is the collection of an expressive language sample (ESL) that could be used even with minimally verbal children [54] and could be collected by the parents themselves. One of the main challenges in this

kind of assessment is the standardisation and validation of these measures, but in the past few years there has been a huge interest in developing reliable measures to administer and analyse language-based assessments, and the progress have been outstanding [55].

These aspects will be further discussed in section 1.4. as well as the potential for a non-invasive composite biomarker that could combine many of the above-mentioned measures, to increase the chances of identifying strong and reliable targets of interest for drug discovery. The most evident common issue that motivate research on the above-mentioned diseases, is that there is currently no cure, and only management of symptoms therapies are usually available.

1.3.2 Biomarkers in neurodegenerative disorders

Similar to the field of neurodevelopment, an extensive body of literature deals with biomarker discovery in neurodegeneration; we will not focus on the details of the different biomarkers or candidate biomarkers for each neurodegenerative disorder, as the field is broad and beyond the scope of this introductory chapter and of the project. We will rather concentrate on the main approaches to biomarker discovery, on some of the major neurodegenerative disorders. Identifying biomarkers is also a prominent matter in neurodegenerative disorders, and early diagnosis is one of the biggest challenges in neurodegeneration, especially to tackle progression of the disease at the earliest stage possible. Therefore, it is vital to identify different measures that could help improve clinical diagnosis when symptoms like cognitive decline are common and overlap between different neurodegenerative diseases making a clear diagnosis very difficult using current methodologies. Different kinds of biomarkers are studied with this purpose, particularly prodromal biomarkers [56]. These are measures that are detectable early in disease progression but where the disease cannot be unambiguously diagnosed. On the other hand, prognostic biomarkers are useful to predict the rate of progression of a disorder and the risk of further development, as well as helping with differential diagnosis[57]. Moreover, biomarkers that help in determining the stage or phase of a disease play also a fundamental role. Finally, therapeutic biomarkers that serves as a measure to estimate the efficacy of therapies and treatments [58]. Hansson et al. [59] reviews the latest approaches in biomarker discoveries and amongst the most important he highlights some techniques such as Positron

Emission Tomography (PET) scans, successfully used for example to detect amyloid- β and tau pathology in Alzheimer's Disease (AD) even years before the development of the disorder as recent findings suggest that the accumulation of A β begins around 20 years before the development of AD.ⁱⁱⁱ PET scans have also been used to determine the levels of dopaminergic nerve terminals in the basal ganglia which are reduced in Parkinson's Diseases (PD) for example, amongst other disorders. Magnetic resonance imaging (MRI) is another very common brain imaging technique which is routinely used to detect and assess neurodegeneration, and to study brain atrophy [61]. MRI was successfully used to detect a promising early sign of Fragile X-Tremor Ataxia Syndrome, as the Middle cerebellar peduncle (MCP) width seems to be decreased in premutation carriers who can develop FXTAS [62]. Fluid biomarkers also play a fundamental role in neurodegeneration, particularly proteins that are detectable in CSF, such as α -synuclein^{iv} which is usually decreased in PD, and Multiple System atrophy (MSA) [58]. However, CSF is a very invasive fluid to collect as mentioned in 1.3.1, therefore many recent researches are focusing on trying to detect expression of some of those same proteins in different parts of the body. Recent evidence suggests that α -synuclein can indeed be detected in the peripheral nervous system through skin biopsies that contain nerves [63]. Amongst the proteins that can be detectable both in CSF and in blood samples Neurofilament light chains (NfL) is consistently altered in different forms of

ⁱⁱⁱ Tau and A β are two of the key proteins associated with neurodegenerative diseases: tau is a microtubule-associated protein and is mainly found in axons, playing a fundamental role in microtubule assembly and stabilisation. A β , derived from the amyloid precursor protein (APP) is present in neuronal synapses and plays a fundamental role in neural growth and repair. Pathological expressions of tau are believed to play a fundamental role in many neurodegenerative disorders, while accumulation of A β in the brain is believed to be an early sign of toxic accumulation connected to Alzheimer's Disease, although the A β mechanisms responsible for dementia are still unclear. However, using PET scan, a neuroimaging technique that uses radioactive drugs to detect atypical metabolic functions in the brain, researchers were able to detect deposits of A β in the medial parietal and frontal cortex long before other cortical regions were affected.

59. Hansson, O., *Biomarkers for neurodegenerative diseases*. Nature Medicine, 2021. **27**(6): p. 954-963. 60. Chen, G.-f., et al., *Amyloid beta: structure, biology and structure-based therapeutic development*. Acta Pharmacologica Sinica, 2017. **38**(9): p. 1205-1235.

^{iv} α -synuclein is another key protein associated with neurodegeneration pathology, is expressed mainly in neuronal synapses. Pathological modified α -synuclein aggregate causing neurotoxicity in several different neurodegenerative disorders, including Parkinson's Disease, Lewy Body dementia and multiple system atrophy (MSA), making differential diagnosis at early stages very difficult. Some evidence suggests that aggregation of this protein could start in the olfactory bulb or the dorsal part of the vagus nerve and even in the peripheral nervous system. 58. Koníčková, D., et al., *Biomarkers of Neurodegenerative Diseases: Biology, Taxonomy, Clinical Relevance, and Current Research Status*. Biomedicine, 2022. **10**(7): p. 1760. 59. Hansson, O., *Biomarkers for neurodegenerative diseases*. Nature Medicine, 2021. **27**(6): p. 954-963.

neurodegeneration. ^vNfL reflects axonal damage and is usually increased in Amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD) and parkinsonian disorders, as well as AD. In AD evidence suggests that increased levels of NfL can be found in AD as early as 15 years before the development of symptoms [64]. Additionally, higher levels of NfL seem to be correlated with a faster progression of the disorder and higher brain atrophy rate [65]. This makes NfL a good candidate to assess the status of the disorder as well as the efficacy of treatments that can normalise NfL levels [58]. However, it is considered a nonspecific biomarker as increased levels of NfL have been detected in several different neurological disorders. Another promising blood-based biomarker discovery technique is proteomics untargeted analysis (commonly referred to as proteomics), which has shown potential for identification of several proteins that are altered in a disorder such as Fragile X-Tremor Ataxia Syndrome (FXTAS) [66], where several fluid based biomarkers are being investigated such as altered lipid metabolism, found in a longitudinal study of FXTAS patients [67], as well as elevated expression of *FMRI* mRNA[68].

^v Neurofilament Light Chains (NfL) are cytoskeletal neuronal proteins present mainly in axons. Damages to axons causes the release of NfL in CSF and blood, therefore it represents a perfect candidate to detect different kinds of axon damages in different pathologies. 58.Koníčková, D., et al., *Biomarkers of Neurodegenerative Diseases: Biology, Taxonomy, Clinical Relevance, and Current Research Status*. Biomedicines, 2022. **10**(7): p. 1760.

Disease	Symptoms	Biomarkers
Alzheimer's Disease (AD)	Memory issues Word finding difficulty Executive functions difficulties Decision making difficulties	• PET scans and MRI • Blood-based such as tau Amyloid-beta, NfL
Parkinson's Disease (PD)	Tremor Rigidity Slowness of movement Mild memory and thinking problems Sleep problems	• Blood and CSF based such as alpha-synuclein, NfL • PET scan for dopaminergic level
Amyotrophic Lateral Sclerosis (ALS)	Twitching and cramping of muscles, especially those in the hands and feet Loss of motor control in the hands and arms Impairment in the use of the arms and legs Tripping and falling Persistent fatigue	• PET scans and MRI • Blood based such as NfL.
Frontotemporal Dementia (FTD)	Increasingly inappropriate social behaviour Loss of empathy and other interpersonal skills Lack of judgment Loss of inhibition Lack of interest, also known as apathy Compulsive behaviours such as tapping, clapping, or smacking lips over and over A decline in personal hygiene Changes in eating habits	• PET scans and MRI • Blood based such as NfL.
Fragile X-Associated Tremor Ataxia Syndrome (FXTAS)	Tremor, gait ataxia, executive dysfunction and dementia, executive dysfunction, peripheral neuropathy	• Proteomics identified several proteins that changes compared to controls • Elevated expressions of FMR1 mRNA • Decreased MCP width

Table 6: Biomarkers in neurodegenerative disorders.

The table summarises the main progress in biomarker discovery in some of the most researched neurodegenerative disorders.

The progress made in identification of measures that could be used as biomarkers has been outstanding, and potentially blood-based biomarkers will be further explored as many markers are detectable in blood reducing the difficulties and costs compared to collecting CSF. Despite overlapping symptoms and similar mechanisms it is fundamental to investigate these potential measures to differentiate among the many neurodegenerative disorders, as differential diagnosis still remains one of the biggest challenges in neurodegeneration [69]; each condition presents with its own development and progress, and different conditions need different treatments, targeted for the specific needs in order to improve efficacy of new therapies. Combining different measures could represent a viable approach to tackle this challenge and to develop new therapeutical targets. Particularly, assessing pathological language patterns show promise, especially when combined with other type of measurement, such as

molecular analysis or electrophysiology. Language assessments are becoming predominant in detecting signs of neurodegeneration in the main neurodegenerative diseases such as AD [70], PD [71], FTD [72], and primary progressive aphasia (PPA)[73] and they all present with specific characteristics and patterns that potentially could help in take a step forward into effective ways to make differential diagnosis. Pathological language signs could often be detected earlier than other symptoms, as language is a complex apparatus dependent on a delicate equilibrium of several different brain mechanisms [74]. The pathogenesis of each disorder could vary, hence understanding what causes potential language impairment, could help shed a light on which brain area for example is most affected, or which pathway and at which stage of the disease. Consequently, this could bring forward drug discovery research and help target interventions. Moreover, language assessment can be cost effective, non-invasive, and easily reproducible which could help tackling the lack of diagnostic instruments especially in countries where CSF collection or brain imaging are unaffordable or uncommon [75]. In their 2023 review Garcia et al.[75], summarise the main findings in speech and language markers for neurodegeneration and they emphasise the need for further studies, particularly investigating languages other than English, as linguistic symptoms could vary across languages, depending on the morphosyntactic, phonetic or semantic complexity of the language [75].

Disease	Speech/language deficits	Neural correlates
Alzheimer's Disease (AD)	Semantic deficits Simplified grammar Figurative language difficulties	Hippocampal, temporal and temporo-parietal regions
Parkinson's Disease (PD)	Dysarthria Action-verb and morphosyntactic difficulties	Basal ganglia, thalamus, motor cortex, temporal lobe
Primary progressive aphasia	Impaired motor speech Semantic difficulties Word-finding and phonological difficulties	Inferior frontal and motor regions Anterior temporal lobe Parieto-temporal regions
Frontotemporal Dementia (FTD)	Prosody difficulties Reading difficulties Social concept difficulties	Fronto-insulo-temporal regions

Table 7: Language deficits in neurodegenerative disorders.

Overview of the main language deficits in neurodegenerative disorders. based on Garcia et al., 2023 [75].

As shown in Table 7, identifying the deficits specific to a certain disorder could help identify the affected brain region, hence target more precise interventions. Therefore, we believe that studying language and trying to understand the mechanism underlying pathological patterns, could represent a valid tool to help finding reliable and effective measures to tackle neurodegeneration at its earliest stages, especially if this measure is combined with other candidate biomarkers.

1.4 . Language measures: language as an assessment measure and a candidate biomarker.

We briefly mentioned in 1.3.2 the emergence of the study of language in clinical settings as a potential outcome measure for neurodevelopmental disorders, and the study of pathological patterns as potential biomarkers of disease progression in neurodegeneration, and how language assessment combined with other measures could represent a powerful asset in the quest for new therapeutic targets. Investigating language studies in literature led us to the hypothesis that common linguistic features and mechanisms could be disrupted in both FXS and in FXPCs and this could help us better understand underlying mechanisms responsible for language impairments. In this section we will further explore these concepts, first by giving an overview of the available literature on language development patterns in FXS and on potential pathological patterns in FXPCs. Then, we will explore language in healthy aging and in neurodegeneration.

Language in FXS

The study of language and how it is connected to the mind has always fascinated researchers all over the world, and understanding language development is still one of the most discussed topics in cognitive sciences. Ever since Psammetichus, an ancient Egyptian king did what is now considered the first scientific experiment to understand language^{vi}, the linguistic ability of children to learn the faculty of language has always interested researchers [76]. Today the study of language development is a prominent applied research topic, it is now mixed

^{vi} This experiment has been reported by the Greek historian Herodotus, and the investigation was focused on finding out who represented the original “human race”. King Psammetichus ordered that two children were to be isolated and brought up by shepherd’s who were to speak to them. The idea was that the children would eventually start to speak on their own the original human language. Herodotus reports that one of them eventually said a word that sounded similar to the Phrygian word for bread and therefore the king had to declare that Egyptians were the second oldest race and not the oldest. This experiment today is quite cruel and unethical, but it highlights the fascination about uncovering the language development mechanisms since the ancient times 76.Hoff, E., *Language Development*. 2005: Wadsworth, Inc. Thomson Learning. 492..

with many different aspects, and it is particularly flourishing in neuroscience as it can be used to uncover several mechanisms of brain functioning and development. Empirically, in early development, evaluating language or communicative non-verbal abilities is one of the common measures to assess the general health status of the child. Delays, or impairment in the first stages of the development could be signs of potential learning disorders or other issues that concern the overall developmental stage. The study of language in neurodevelopmental disorders therefore plays a fundamental role in research when assessing the general physiopathology of a specific disease, to better understand the underlying mechanisms responsible for the disorders. In FXS language is a faculty that is affected both in males and females and for the purposes of this project it is important to describe the linguistic profile of FXS and the investigative approaches of research toward the field of language development, from the earliest stages of development to adulthood.

Early development

Most research on language in FXS have focused on childhood and adolescence. Despite the lack of comprehensive studies on infancy and toddlerhood, some research has been able to establish certain milestones for language development in FXS.

Delays are present before the third year of age, the first word is produced around 26/28 months of age, while in typically developing children it emerges before the 18th month of age. Around 9 months of age, typically developing children begin to communicate [77]. Eventually they produce their first word and then the length of their utterances continue to grow [78]. Additionally some retrospective video analysis of children of 9 to 12 months of age have found non-verbal communication delays in FXS as predictors of a slower language growth and some studies reported mothers saying that their children were stuck at a non-verbal stage at 18-36 months or produced few words [79].

Only a few studies adopted a longitudinal perspective to gain a better understanding of the different developmental stages. A study in 2015 [80] assessed language development in 13 male toddlers and infants with FXS using a series of standardised tests and parent reported questionnaires and interviews. They assessed children at 4 different time points (9,12,18, and 24 months of age) comparing results with 11 controls (12 months of age). They were able to see

patterns of delays very early in infancy, and to identify a significant delay in language development related to expectation levels for chronological age at 18 months, compared to typically developing controls. As the first study which directly assessed toddlers and infants, and correlated results with parents' interviews, it provided insights on the earliest stages of language development in FXS and contributed to identify parents reports as a reliable tool to assess language skills in children with intellectual disabilities.

The studies mentioned above focused on boys with FXS, with very few studies examining language in toddlerhood and infancy in girls with FXS, finding a much higher delay in boys [79]. Consequently, further comprehensive research on the very first stages of communication development in FXS are needed, especially with an eye toward the gender difference, which is still less explored.

School aged children and adolescence

Most research on language development of FXS have focused on childhood and adolescence. As mentioned earlier, some studies revealed that many boys with FXS remain essentially non-verbal as they entered adolescence [81]. For those who start to speak, both expressive and receptive language continue to grow even if at a slower pace than typically developing children. Results are again controversial, some identified receptive language as a strength others as a weakness [82] therefore, it is worth describing results obtained both on language comprehension and on expressiveness.

Receptive language

Receptive language refers to the ability to understand spoken or written language, with vocabulary comprehension specifically referring to “words comprehension” and “syntax and grammar” referring to sentence complexity comprehension [83].

In some studies receptive vocabulary skills of boys with FXS, measured with standardised tests, have been found in line with non-verbal cognitive skills and at a similar levels of typically developing peers [84] [85].

In other studies boys with FXS performed significantly worse in vocabulary comprehension than matched peers, even when correlated to non-verbal cognition [86]. When controlling for a diagnosis of autism, some research failed to find a significant difference between children with autism and those with FXS only [87], while in other studies children with both conditions performed worse.

Interestingly, in a study receptive language's low scores have been considered a marker for autism symptoms in children with FXS, because children with both diagnosis performed worse on all the measures, while children with FXS only, receptive language was a relative strength [88].

Particularly grammar and syntax seem to be below the expected age level in young adolescents with FXS [86, 89, 90]. Interestingly, they showed lower scores on standardised tests of syntactic comprehension than their matched peers [86, 90], while older adolescents and young adults performed similar to the typically developing peers [84]. This could mean that maybe individuals with FXS eventually reach their peers level growing up, filling the gap present during childhood and early adolescence.

On the contrary, other studies failed to detect differences with typically developing adolescents [84].

Little is known about language development of females because many studies excluded girls when selecting participants. In one study, males performed worse than females in certain subsets of standardised testing and similar in others, suggesting an overall delay and poorer ability in males compared to females [84]. In general, the studies mentioned above included small sample sizes of females, showing evidence for lower scores on developmental receptive syntax compared to typical development, regardless of the gender, but more research is needed to draw decisive conclusions [91].

Expressive language

Although all areas of language performances are potentially impaired in FXS, expressive abilities seem to be more negatively affected than receptive ones [92]. One of the issues when analysing expressive language is the type of assessment used. For a long time, expressive abilities have been measured with standardised tests, which has proven not suitable for individuals with FXS, because of the high within-syndrome variability. Consequently, recent studies have focused on identifying reliable measures to analyse Expressive Language Samples (ELS), a useful tool to assess spontaneous speech, avoiding the burden of long and challenging standardised tests.

Expressive vocabulary growth appears to be in line with non-verbal cognition levels, when measured with standardised tests, and when a child starts to speak their vocabulary continue to grow at a slower pace than matched peers [83].

The aspect on linguistic abilities which appears to be more affected in development is syntax and it is also the more explored in literature.

In the early literature of the 90s findings related to expressive language sampling are inconsistent and based on overall measures of the Medium Length Utterance (MLU) and IPSyn score (Index of Productive Syntax), which are general assessments, incapable of giving a more specific measure of the different aspect of syntactic abilities. This contributed to the controversial results, where some failed to find a significative difference between expressive syntax of pre-schoolers with FXS and their typically developing peers, while others reported poorer performances of children with FXS [93].

In the last 15-20 years the situation has changed, and research have focused on more specific measures of morphosyntactic skills.

Another important feature in measuring expressive language is the different type of sampling used, meaning that the context can affect language production [94] (i.e. the structures and cognitive resources used for narrative or descriptive tasks is not the same). Four recent studies have contributed to the advance in assessing expressive language abilities for the results and the methodology used, with a specific focus on grammar and morphosyntax. In 2010 Finestack and Abbeduto [93] evaluated the expressive language skills of a group of 24 highly verbally expressive adolescents (from 11 to 23 years) with FXS and Down syndrome (DS), compared to a group of Typically Developing (TD) peers.

They collected narrative language samples which elicit more complex utterances compared to conversational ones. They analysed individuals' performances using different measures of oral expression for lexical diversity and syntactic abilities, and a language analysis coding which assess performances across different grammatical domains [93].

They also included 5 females in the group who outperformed males in the oral expression measures. Interestingly, when females were included in the overall measure, the FXS group outperformed the TD one, but when females were excluded no big differences between the two groups were found. When narrative language samples were analysed with the coding system, the FXS group had general poorer performances compared to matched peers, particularly when measuring the use of negative sentences and verbs. No difference was found in the subcategory of nouns production (indefinite pronouns, personal pronouns, noun modifier etc) between TD and FXS groups [93].

These findings are close to two other studies on expressive language abilities whose scores were based on conversational language sampling.

Price et al. [95] and Roberts et al. [96] analysed the language skills of younger children (2 to 7 years of age) and the FXS group scored overall worse than the control group, including nouns production but especially when measuring verbs production (regular or irregular past tense, -s third person, etc). While there is a discrepancy in finding relative to nouns production, the lower score in verbs production is present in all the studies, probably due to the different nature of language samples or to the age difference, meaning that younger children eventually reach a comparable performance of typically developing children concerning nouns, but the difficulties in verbs production remain. These findings are particularly important for the purpose of this study, because the gap between verbs and nouns in expressive language has been related to underlying difficulties in the language processing mechanism [97].

Following this line of research another study investigated expressive morphosyntax of boys with FXS, with and without autism to verify the existence of specific syntactic problems associated with FXS [98]. As mentioned earlier, expressive syntax seems to be particularly weak in FXS, but the origins of this impairment are still unknown. A common pattern seems to attribute more difficulties in verbs production than nouns, consequently their aim was to examine this difference by measuring token frequency of nouns and verbs in conversational speech samples, to gain a more specific syntactic measure of the FXS language phenotype.

They analysed speech samples of highly verbal individuals (more than 40 words) of 16 years or less. The speech samples were analysed from the ADOS administration (Autism Diagnostic Observation Schedule) [98] and they consisted in recordings of different kinds of interactions with an examiner. The analysis consisted in the count of produced verbs morphemes (past tense, -s singular, finite “be” copula, auxiliary be/have) and nouns morphemes (a, an, the, possessive’s, the overall production of morphosyntax related to nouns).

Results showed overall impairment in morphosyntax production of both groups with FXS, regardless of the ASD status, but there was no direct evidence of a more impaired production of verb’s morphemes (except for some subcategories), children with FXS showed greater difficulties in articles productions too for examples.

This study was of a quantitative nature, and it measured the number of morphemes produced by children in some specific context, it did not account for a potential qualitative difference in verbs and nouns productions which has been the focus of various studies in expressive language in children with language disabilities, and it can be related to potential difficulties in the retrieval of verbs compared to nouns. Following this line of research could be a useful path to understand potential problems in the mental lexicon organisation of individual with FXS.

To summaries, these various results on both language comprehension and production are controversial but all the studies found a huge within-syndrome variability whose origins are still unknown. Only recently, some researchers have tried to address this issue. In the next section we are going to highlight the most important findings on this subject.

Why language is impaired? Predictors of language growth and language specific factors

When learning a foreign language, individual variability is a commonly observed phenomenon. For this reason, research on foreign language acquisition have focused for a long time on identifying cognitive and environmental factors contributing to the differences. As there is a huge within-syndrome variability in language development of children with FXS focusing on potential factors contributing to these differences in language growth could be a promising path. Predictors of linguistic abilities can be varied, including cognitive factors such as working memory and environmental family-related factors.

To date, only two cognitive factors have been identified as predictors of language abilities in FXS: phonological memory and visual attention.

Phonological memory skills refer to the ability of retaining and rehearsing the phonological information^{vii}, while verbal working memory refers to the ability of manipulating the information [99]. Following this distinction Pierpont et al. in 2011 identified for the first time phonological and verbal working memory abilities as predictors of specific linguistic skills in boys with FXS, but not in girls. Particularly, phonological memory was connected both to better

^{vii} Phonological information refers to the ability of understanding, parsing, and retaining sound segments (phonemes), while verbal working memory manipulation involves the ability of understanding the meaning of the sounds and connect them to the correspondent letters in a specific language when reading.

vocabulary and syntactic skills in a 2 years follow-up assessment, while verbal working memory was only related to a growth in vocabulary abilities in boys [99]. All the language measures were collected using standardised assessment. Two others studies connected word recall to performances in narrative speech sample of boys with FXS, specifically accounting for syntactic performances [100] [101].

In contrast with Pierpont et al., Kover and Abbeduto [91] found a correlation with performances in syntactic abilities in girls in narrative speech samples, but not in conversational ones, probably due to the higher demand of narration compared to conversation, involving a much stronger contribution of cognitive functions. Consequently, they concluded that phonological memory might not be crucial for language development in females with FXS as revealed by Pierpont, but it could be an important factor for language use. This is believed to be a feature of FXS linguistic phenotype in general, [91] as a pattern found both in males and females with FXS [101].

Another cognitive predictor of language abilities identified in FXS is visual attention.

Children with FXS often display problems in attention, especially in disengaging attention from a referent. In 2015 Kover et al. examined visual attention of 13 males with FXS and 11 controls at 4 time points (9,12,18, and 24 months of age) with both direct assessments and parents' reports. Both measures identified early visual attention as predictor of language development during the 2 years of the study, additionally the inability to disengage attention from a stimulus was negatively correlated with language development [80].

Among environmental factors, mother-children's interactions have been considered a factor affecting language development. In a longitudinal study Brady et al. [102] analysed mother-child interactions once every 18 months from the age of 11-216 months in 55 individuals. Their results showed an important role of maternal commenting during interactions, which was predictive of many language variables and outcomes (such as the number of different words used and receptive vocabulary) [25]. However, in another study analysing expressive syntax, maternal responsivity was not predictive of growth in expressive syntax, probably because syntax was not fully developed in the cohort taken into account, or maybe because maternal responsivity is only predictive of vocabulary, as the previous study indicate [103].

Finally, mother-child relationships seemed to be predictive of talkativeness in conversation for 57 male adolescents where changes over time were found over annual assessments, observing an increase in talkativeness and a decrease in lexical diversity and syntax complexity. Children with a closer relationship with their mothers showed less decrease, while individuals whose mothers had a higher level of education, college degree, showed a higher rate of talkativeness in conversation. [83].

Taken together these findings show an important role of both cognitive factors and environment in language development of both males and females with FXS, but further investigation is needed.

Language patterns in Fragile X Premutation Carriers

Historically, studies on the clinical involvement of the *FMR1* gene have focused primarily on individuals with the full mutation because individuals with the premutation were considered unaffected. As mentioned in 1.2.3, in the last twenty years research have demonstrated that carriers could be affected by the compromised functions of the *FMR1* gene while ageing.

One of the most alarming consequences is the early onset of FXTAS.

As well as in many other neurodegenerative disorders one of the main challenges is the detection of symptoms at a pre-clinical stage, to identify potential individuals at risk before the development of the disease.

To this aim, linguistic abilities have been studied in ageing population because detecting potential difficulties in language production has been considered a hallmark of cognitive decline for example in PD[104, 105] or in the preclinical stage of AD [106].

Disfluencies are common in everyday language but at a certain rate they can be reflective of pathological outcomes, and are usually defined as “*disturbances in the flow of information that impair continuity of language sequencing and information content*” [7], such as repetitions, self-corrections, pauses etc.

That is why a few studies have recently used such a measure to potentially identify individuals at risk of developing FXTAS among FXPCs.

The first study which identified pathological disfluencies in language in pre-mutation carriers has been carried out in 2013[7].

They collected a 5-minute expressive language sample where female carriers showed a more disfluent linguistic profile than the control group. To rule out the

effects of raising a child with a neurodevelopmental disability, the control group was made up of mothers of children with autism spectrum disorders (ASD). The FXPCs group displayed a more disfluent speech in all the measures with a clear impact of age, leading the researchers to the hypothesis that it might represent a pattern of ageing with a premutation, potentially indicating the risk of developing FXTAS or a milder phenotype. They explained this pattern as a marker of difficulties in planning, involving impairment in executive functions. And they were able to correlate the disfluencies with the CGG repeat length (low and medium range seemed to be the highest risk factor and executive functions impairment) [7].

In 2017 linguistic and cognitive abilities were used as automated screening for FXPCs using mobile devices and machine learning [107]. They investigated language disfluencies in 200 premutation carriers, mothers of children with FXS, and a comparison group of mothers of children with ASD. The linguistic profiles of the premutation group were significantly more disfluent than the control group. They identified a very specific linguistic profile, examining not only the number of disfluencies, but also other features related to the density of language (for example the use of shorter and simpler utterances). They were unable to find significant differences in executive function measures and working memory. However, they detected difficulties in speech planning in premutation carriers that were not found in the comparison group, especially at the beginning and at the end of their speech samples [107].

Subsequently, another study investigated language disfluencies in women collecting 5 minutes of spontaneous speech samples. They again confirmed the existence of a much higher number of disfluencies in the premutation carriers' group, compared to mother of children with other intellectual disabilities, and they also found a correlation with the number of CGG repeats [8].

In line with the other research, they were unable to trace back the disfluencies to inhibition deficits and they hypothesised a potential impairment in some language specific process, rather than a more general executive function mechanism.

This evidence shows that the hypothesis connecting language disfluencies to deficits on executive functions is yet to be confirmed, on the contrary these studies consider the hypothesis misleading, pointing out that a lot of other factors

are involved, especially some language specific process like lexical access, the process by which we find the right words to speak properly [8].

Furthermore, a very recent longitudinal study examined the standard 5-minutes speech samples of female carriers over 8 years [108]. They identified increasing word retrieval difficulties in the premutation group with a clear effect of age. Until then no studies had investigated language disfluencies in speech of man with the premutation. Finally, a very recent study investigated to what extent language patterns differ between males and females and whether language production represented a good predictor of FXTAS symptoms with a 5-minute language sample. Males and females did not show a different language pattern; however, language production was a good predictor of FXTAS symptoms. However, this study differs from all the others as it recruited participants with symptoms of FXTAS, making it possible to correlate language measures with those symptoms [109].

In this project, we are going to address this issue, and our aim is to examine a language specific process, lexical access, and retrieval, which has been indicated as a factor contributing to disfluencies in language, as to date factors potentially leading to the mentioned language disfluencies have not been properly investigated.

Linguistic outcome for clinical trials

Currently, there are no approved treatments or cures for FXS or FXPAC and FXAND. The ineffectiveness of some clinical trials attempted so far, has been correlated to the lack of reliable endpoints to be used in clinical trials, and language outcomes appears to be an attractive measure because changes in communicative skills have a direct impact in real life [110]. The identification of reliable outcome measures has been a major concern of several studies, particularly results from a report in 2013, led by a pool of scientists and clinicians, identified potential endpoints which included different kind of measures, cognitive, behavioural, biomarkers and linguistics outcomes [111].

Amongst the linguistic assessments examined (standardised tests, laboratory-based tasks, caregivers report etc), Expressive language samples (ELS) measures appeared to be the most promising instrument, because they are applicable under several different circumstances and ages. Additionally, progress in research on

ESL in FXS has led to the identification of potential standardised way of analysis and codification of results.

A follow-up report in 2018 [112] still identified ELS as very promising, because as opposed to standardised tests a series of psychometric works have been conducted in ELS resulting in confirmation of the potential usefulness of these measures in clinical trials. This report mentioned the only study which has tried so far to focus on a potential impaired linguistic process, rather than measuring the amount of language produced, as both standardised tests and ELS measures tend to rely on. This study investigated fast mapping, an associative process which children use in the earliest stages of vocabulary acquisition to learn the association between a referent (objects) and linguistic labels (words) [92]. This line of research is considered promising in clinical trials because it can be applied to minimally verbal individuals, it can be computerised and administered remotely, and it can be applied to measure short-term learning in real time. However, there are no psychometric validated works for these kinds of outcome measures to date [112].

ELS measures are undoubtedly valid and need to be further explored, nevertheless there are still some issues with the use of these kind of language samples: some structured testing context have been identified, such as the impact of the different kind of samples collected (Narrative or Conversational for example) [94], but inconsistencies can still be present in the transcription of the linguistic samples. Additionally, these kinds of measures do not account for the process but only for the outcomes, and more importantly it can only be applied to highly verbal individuals.

In this project we aim to address this matter, by trying to investigate the process that could be responsible for the abnormal language patterns identified in FXS and FXPCs. We believe this could be a valuable instrument for several reasons:

- a) First, the tasks we developed are highly reproducible and can be adapted to different age groups and it can be used on individuals with low verbal outcomes.
- b) Secondly, the process we aim at investigating, lexical access, is still relatively unexplored in individuals with intellectual disabilities. However, it holds the potential to uncover fundamental mechanisms responsible for language production and reception, both in neurodevelopment and in neurodegeneration, as it involves a lot of cognitive factors, and it activates a

number of different brain areas and language pathways. Therefore, focusing on a specific potentially impaired brain mechanism, could be a valuable instrument to test new treatments [113].

c) Lastly, the same experimental tasks could be applied both to FXS as a potential outcome measure and to FXPCs because evidence shows that the same mechanism could be an hallmark of cognitive decline in adulthood, thus it could be used to screen population at risk of cognitive decline [107] and to identify targeted interventions or treatments before the development of the most severe symptoms. Importantly, language has already been used as an outcome measure in FXS, particularly in a trial which showed that language, measured with a set of standardised tests, and scores of daily functioning, can be improved in adults males with FXS using a phosphodiesterase-4D (PDE4D) allosteric inhibitor (BPN14770) [114]. These results confirm the validity of using language as an endpoint in clinical trials.

The evidence collected so far show controversial results in the literature concerning language development in FXS and a lot still needs to be done.

Larger scale, longitudinal research is needed to gain a better descriptive profile of the different stages of linguistic abilities of individuals with FXS, a lot is still unknown about the early stages of language development and the role played by environmental as well as cognitive factors and certainly, there is a clear need to expand research on predictors of language growth to address the fundamental issue of the large individual variability, as well as studies targeting similar sample sizes of males and females. Additionally, it is also necessary to conduct more research on premutation carriers as very little is known about this condition which affects 1 per 113-259 women and 1 per 260-813 men [7].

What is clear is that one of the biggest issues of growing up with the neurodevelopmental disorder FXS is to be able to express oneself through language, at the same time this ability seems to be affected on FXPCs in adulthood as well. It is striking that only a few studies focused on language specific processes rather than other more general cognitive functions, especially because evidence from studies on FXPCs seem to point to the direction of the existence of a language specific impairment connected to the compromised functions of the *FMRI* gene.

That is why we believed that is vital to address this matter and to investigate the existence of potential common issues in lexical access and retrieval, both in FXS

and in FXPCs. Particularly, in children this could reflect difficulties in the organisation of the mental lexicon and shed a light on potential language specific mechanism or language pathways that are disrupted in the brain. In FXPCs, difficulties in lexical access and retrieval could be markers of cognitive decline. We will focus on the difference between verbs and nouns production, identified as a difficulty both in children with developmental disabilities [115] and in adults at risk of neurodegenerative disorders [106].

Thus, language could be a very interesting starting point as it is a non-invasive assessment, it is cost effective, and it can tell us a lot about the general health status of the brain. The details about the linguistic assessment will be discussed in Chapter 2.

However, we believe that only studying one measure is not enough to build a strong candidate for biomarker discovery in *fMRI* research.

Therefore, our plan is to combine language with a fluid biomarker. Based on recent literature fluid biomarkers are the perfect candidate for research both in neurodevelopment and in neurodegeneration.

In this project we were only able to apply the linguistic assessment on FXPCs; this matter and the reasons behind this choice will be further discussed in the next sections.

1.4.1 Research on language in healthy aging and neurodegeneration.

The origins of the study of language in the brain.

Fascination about language processing and functioning has always been present in the human nature. We mentioned the example of the very first experiment performed by the Egyptian Psammetichus, and we could name a few others that despite the rudimentary and quite unethical approach are witnesses of the natural human curiosity about uncovering the language acquisition process. The field of language development as we know it today was born in the 1950s and the time two main theories were predominant:

- Behaviourism, a theory who asserts that changes in behaviour are strictly dependent on a precedent action and that every kind of behaviour can be explained with reasons external to the mind [116].
- Cognitivism, a completely opposite theory as which assumes that it is fundamental to understand how the mind works to understand behaviour [117].

Before the 1950s Behaviourism was predominant in psychology and related fields, but after the Cognitivist Revolution everything changed, and the study of language played a central role in this revolution. Behaviourism emphasises a system of positive or negative reinforcement to shape behaviour. However, this theory was challenged by finding that children acquire language and grammatical rules regardless of the positive reinforcement. This led to the rise of the new emerging cognitive sciences focusing on the importance of understanding how the mind works to explain behaviour. However, cognitivism did not flow into a unique set of ideas, instead it gave rise to many different theories on language acquisition [118]. The Behaviourist theory is now considered outdated, as the believe that language is exclusively acquired by a mechanism of imitation and reinforcement does not seem to account for most language acquisition processes. All current theories agree on the idea that some part of language acquisition is innate, but they differ on the degree of what is innate and what is learned from experience. Below a brief overview of the different theories born after the cognitive revolution.

- The nativist theory, proposed by Noam Chomsky, proclaims that the ability of learning language is biologically determined and is based on the fact that even children with poor exposure to language are able to learn language, without any system of reinforcement; as the pioneer studies on the several *enfant sauvages* revealed, what matters is the time frame of the acquisition of language, within what Lennenberg called the critical period [118].
- The modern alternative to the nativist view is the interactionist theory, having one of the major exponent in Braine, this theory supports the idea that some characteristics are innate in language acquisition, but the kind of input the child receive is also crucial [76].
- Lastly, it is worth mentioning the emergentist theory supported by MacWhinney, Bates, and Goodman, asserting that the interaction between biology and the environment is what matters. This belief gave rise to many modern theories on language development such as the connectionist and the neural network models, which shift the focus of the debate from innatism versus environment, to the acceptance that some part of language acquisition are innate, but we need to investigate what is innate [76].

These latest theories will be discussed later in this chapter as they offer some useful perspectives to the understanding of the conceptual background of this study. All these assumptions have been fundamental to the modern study of language, and their main principles are important to understand the implications of how studies about language processes are designed and interpreted. One of the most debated aspects in language studies is whether the language apparatus is modular or not. Innatism postulates the existence of a language specific module based on the assumption that knowledge is itself innate. Their theory is based on the idea that all languages in the world share similar characteristics, and what they call a Universal Grammar, which makes possible to extract innate information, common to every language in the world, to be declined in the different languages. This modularity theory postulates that the mind is equipped with a separate and specific module dedicated to language, that Chomsky referred to as the “mental organ” [119]. These assumptions are a part of a larger debate in psychology, questioning whether there are different cognitive abilities for different skills or whether there is a general cognitive ability declined to each different task. Therefore, the modularity theory is not specific to language, but it involves all cognitive abilities. The alternative to this theory is the idea that many other general skills contribute to language acquisition such as memory skills, ability of extracting symbolic representation, pattern analysis and so forth [76]. Connectionism is part of this latest approach as it assumes that knowledge is organised in processing elements called nodes that are activated, depending on experience, through spreading in a network of connected nodes. This theory explains how an individual learn different kind of behaviours depending on the circumstances, and how to extract patterns and regularities from experience [120]. Despite being close to behaviourism in terms of the importance attributed to experience, this theory can highlight the relevance of the interaction between experience and what is innate, as for each node or for each network to be activated, and for the individuals to extract regularities, there must be something innate to be extracted. That is for example how a baby learns how to extract statistical pattern and regularities from the linguistic input they receive, drawing on some sort of innate knowledge they already have [76]. All these different theories and approaches are fundamental in the study of language, not only in language acquisition and development, but also when it comes to study language impairment or when language difficulties arise, for example because of brain

injuries, neurological disorders or neurodegeneration. The Cognitive Revolution indeed, gave rise to the naissance of discipline such as psycholinguistic and neurolinguistic, devoted to the study of the connections between mind and language on the one hand, and between brain and language on the other. Neurolinguistics is the study of neural mechanisms underlying language. It is considered a multidisciplinary field generating from the study of brain injury, aphasia and experimental methodologies, guided by the view that language is organised into structural components each directly connected to a different neural region. Psycholinguistics instead puts together psychological methodology and theories to understand linguistic processes. The main interest is to understand how language works and how communication is achieved through language [121]. Combining the two disciplines to study language deficits could be an asset in disorders affecting language as well because they can provide an interesting and non-invasive approach to understand the underlying mechanism of action of certain disorders, particularly in neurodegeneration. Traditionally the main brain areas associated with language are Broca's and Wernicke's Areas, the first one being correlated to language production and the latter to language comprehension. Modern neurolinguistic approaches tend to involve in language processes neural complex systems instead of only separate areas. Therefore the emergent theoretical framework that came out of the study of aphasia suggests that the temporal lobe is involved in the meaning of words, while the role of the superior temporal gyrus is to process the auditory aspects of speech, the posterior temporal areas instead act as a integratory buffer for articulatory and auditory patterns, and lastly the inferior frontal gyrus is involved in syntax and articulation planning and in some processing for selecting words [122]. In the modern era of neurolinguistics, the development and advances of new tools to have a window into the brain, such as functional MRI (fMRI), TMS, ERP, MEG and DTI, played a fundamental role in advancing knowledge about language processing ^{viii}. To mention an example of one of the major issues in the current debate about language, the ability to

^{viii} fMRI: functional magnetic resonance is a brain imaging technique that detects changes associated with blood flow. TMS: transcranial magnetic stimulation is a technique to stimulate nerve cells in the brain through the use of magnetic fields.

ERP: event related potential concerns the detection of fluctuation in the brain's electrical field due to the presentation of visual or auditory language stimuli 123. Samar, V.J., *Evoked Potentials*, in *Encyclopedia of Language & Linguistics (Second Edition)*, K. Brown, Editor. 2006, Elsevier: Oxford. p. 326-335.

MEG: magnetoencephalography is a technique that measures the magnetic field produced by the brain electrical activity.

DTI: diffusion tensor imaging is a technique that studies the movement of the water molecules in the brain and can detect damages to the white matter pathways.

better map brain regions and networks with modern fMRI, is now challenging the idea of modularity of language, because experimental evidence suggests that the different component of lexicon, grammar, syntax and so forth, do not recruit just one brain area, but they rather involve a broader network [122]. Additionally, they show that the stream of information is interactive and not unidirectional, influencing the different stages of information flow, as cascades lexical access models suggest. Theories of lexical access will be discussed in the next sections. While using psycholinguistic experiments to understand language production and reception could shed a light on what kind of linguistic process are impaired in certain disorders, the knowledge of the neural network associated with the different processes could in turn help investigate the efficacy of potential therapies. As we will explain in the methodology section 2.3, we will use psycholinguistics experiments to potentially understand underlying mechanisms that can be impaired in a population at risk of developing neurodegeneration, and we will avail of the neurolinguistic theoretical framework to speculate potential implication that this might have in developing therapies and intervention for these kinds of disorders, combining the language assessments with molecular measures. The study of aphasic patients provides a theoretical framework that could be used to understand language deficits and the neural network involved. While the study of language acquisition and development helps learn the connection between the environment, the mind and human development. The study of aging and language instead, could inform on deterioration of cognitive processes responsible for language mechanisms. As we age cognitive and sensory changes might cause an additional challenge for language, an already high demanding process. This is true for healthy aging, but what happens with neurodegeneration? In the next two sections we will give an overview of the main processes involved in language in healthy aging (1.4.2) and we will then review the main findings on language in neurodegeneration (1.4.3).

1.4.2 Language in healthy aging

Historically investigation of language areas in the brain started with the study of brain injuries. However, explorations of processes in place during healthy aging can inform on mechanisms underlying language processes, especially in comparison with neurodegeneration. There is now consistent evidence that our

brain undergoes several changes even without neurodegeneration, because healthy aging has numerous implications for the brain as it involves cognitive and structural changes. We know from experimental studies that gyrification decrease [124], and grey matter volume and cortical thickness reduce [125]. Changes are usually described mostly in the prefrontal cortex, but they are expanded to the whole brain. Modifications to the white matter composition are also frequent. A lot of processes are also affected including working memory abilities, attention, and inhibition, while verbal knowledge is usually preserved. Another interesting finding is that hemispheric symmetry is usually less preserved compared to younger adults, and that might be related to a compensatory mechanism [126]. All these structural and procedural changes can lead to changes in language processing compared to younger adults, and it can also involve compensatory and more efficient strategies. Aging by successfully preserving language abilities can be related to the ability of disposing of the necessary neural resources to meet the demand imposed by the several complex tasks to be dealt with regularly. This depends on a lot of factors including, number of neurons, dendritic branching, myelin integrity, working memory, attention on the processing ability side, hence the neural resources, and on features such as word frequency, lexical competition, semantic ambiguity, syntactic complexity on the task demands side [126]. Both language comprehension and production can be impacted in healthy aging: older adults seem to have more difficulties than younger adults in recognising spoken words, in visual words and word and sentence comprehension. Studies investigating word and sentence production also show difficulties in word retrieval for older adults, as showed in tasks such as picture naming or in the tip-of-the-tongue (TOT) experience [126].

The neural basis of word production and reception in healthy aging

In picture naming older adults show increased activity in temporal and frontal cortex, as well as right IFG and bilateral insula in TOT tasks, as well as reduced grey matter correlated with increased TOT in the left insula and anterior cingulate cortex [126].

One of the most important networks in language reception is the cingulo-opercular network. This system, which includes the dorsal anterior cingulate and bilateral frontal operculum, is particularly important in sustained attention task and its activity can predict accuracy. In older adults it seems to be less active,

than in younger adults. Other brain regions are also implicated in word perception, like the bilateral temporal cortex, and interestingly, the occipital cortex is associated with older age. Generally speaking the current state of the literature suggests that older adults might use more brain regions when processing word perception than younger adults, to compensate for the reduced activity in the main network, and that this might affect performance on word retrieval [126]. In this study we are interested in investigating whether potential changes observed in the population of interest are related to cognitive processing enhanced by natural aging, or whether these changes can be caused by a pathological pattern, such as a cognitive decline or neurodegeneration. We will now give an overview about research on language in neurodegeneration.

1.4.3 Language in neurodegeneration

The study of language associated with cognitive decline, dementia and neurodegenerative disorders can be applicable to several domains in clinical studies. Here we will review the current literature on some of the major neurodegenerative disorders which are not primarily affecting language, but that recently have been proven to display impairments in language abilities. We will mainly focus on the different language assessments used to detect anomalies in language skills and we will particularly focus on similar tasks to the ones used in the current study, to assess language production, reception and more spontaneous and less structured language samples, like picture description. The neurodegenerative disorders considered are Alzheimer's Disease (AD), Parkinson's Disease (PD), Corticobasal Syndrome (CBS), Progressive Supranuclear Palsy (PSP), Primary Progressive Aphasia (PPA), Frontotemporal Dementia (FTD), and Fragile-X-associated tremor ataxia syndrome (FXTAS).

Parkinson's Disease (PD)

PD is a neurodegenerative disease caused by loss of dopaminergic neurons in the pathway that projects from the substantia nigra to the striatum that leads to functional impairment of the basal ganglia. The basal ganglia (BG) are recognised as a network of nuclei very important for executive functioning and planning. The basal ganglia and other subcortical structures such as the thalamus, seem to be involved in language processing [127]. The studies of language abilities in PD are particularly relevant to the debate of the embodied cognition framework, where it is questioned whether language specific functions are part

of a broader non-verbal system and especially connected to motor mechanisms. Many studies have focused on the relationship between motor skills and language processing in PD. Despite being the second most prevalent neurodegenerative disorder worldwide, it is still difficult to predict the progression of the disease; neurolinguistic findings can be fundamental to target interventions and therapeutics programs. According to the embodied cognition framework, action verb and motor language are connected to a network that involves stimuli going from the cortex which create a loop with the BG and subthalamic area, and go back to the cortex [127]. Therefore, the BG being affected in PD would cause the specific language deficit. There is evidence of the involvement of the BG system in language operations, however, it is mainly based on studies in PD and Huntington's Disease (HD) and not on direct measures of the BG involvement. Therefore, any inference needs to be confirmed given that these pathologies involves other structures [128]. The embodied cognitive approach to language is based on the evidence that neural sensorimotor systems are activated during language process, challenging the idea that language representation is separated. According to this approach language is strictly connected to action and perception, and there is evidence suggesting that linguistic terms referring to sensorimotor and motor content activate the same neural networks as the bodily experience of the same. What is unclear in this theory is whether this is a product of language processing as a post lexical activation, or whether they are indeed a crucial part and inseparable in language processing [129]. Several studies tried to investigate associations between different aspects of language processing and non-language specific network or perceptual systems. Particularly, most of the studies investigating language in PD found difficulties in action verbs at the semantic and lexical level, with preserved object retrieval. This seems to be related to basal ganglia dysfunction, which would corroborate the embodied cognition framework [127]. The neural correlate of this action deficit are damaged frontotemporal connectivity to the BG network, including the prefrontal cortex, the Broca's area, the anterior cingulate cortex and the subthalamic nucleus [127]. One of the main questions is whether this deficit is *sui generis*, or dependent on a more general executive functions impairment.

Language production

Bocanegra et al. (2015), tested several language skills including, syntax, action verbs, action, and object semantics as well as a series of neuropsychological assessments and executive function assessments. The linguistic tasks included several subtests of the Boston Diagnostic Aphasia Examination (BDAE), such as embedded sentences, action naming, pictures association tests from the Kissing and Dancing Test (KDT), and the Pyramids and Palm Trees (PPT) test [127]. They investigated the above assessments on a group of patients without mild cognitive impairment (MCI)^{ix} and patients with MCI and a control group showing that patient had action semantic deficits even in the absence of MCI, suggesting a very early language impairment. Interestingly, dysfunctions in executive functions predicted object association deficit in both group, but the model did not predict deficits in the action related tasks, which strongly corroborate the impact and importance of the BG in language processing, and the presence of a *sui generis* language impairment unrelated to executive functions. [127]. Action words processing impairment was also found in another study using a lexical decision task in patients without dementia under the effects of levodopa, a dopaminergic treatment, and off medications. The action words processing was decreased in patients without the effect of the dopaminergic treatment, but the process was rescued when patients were under the medication effect, with results comparable to the healthy control group and the performance on the objects word, which was not affected by dopaminergic intake at any stage [131]. In a study assessing lexical access in PD patients compared to controls, using different tasks (picture naming and verbal fluency tasks divided between actions and objects), they did not find significant differences between groups in actions and object naming, but they did find an interesting pattern in the PD group in the qualitative analysis of errors. PD patients had a higher prevalence of circumlocutions to describe action pictures compared to controls, arguing that the nouns/verbs dissociation was caused by the difference in the grammatical class. They also found a correlation with executive functions in the verbal fluency task only, highlighting a potentially major role played by executive

^{ix} Mild Cognitive Impairment (MCI) is the transition between normal aging and early dementia, it affects individuals who experience cognitive decline that is more compared to normal aging but not enough to be diagnosed with Alzheimer's for example. A lot of research is still ongoing to determine the specific characteristics and how to diagnose it correctly. It is an important stage to identify potential individual at risk of developing different kind of dementia, although it is now clear that not all patients diagnosed with MCI develop a more severe neurodegenerative condition. 130. Petersen, R.C. and S. Negash, *Mild cognitive impairment: an overview*. CNS spectrums, 2008. 13(1): p. 45-53.

functions in this task, as previous studies have proven. They argued that verbal fluency tasks are probably not appropriate to measure purely linguistic deficits in this population, as they impose a high demand on executive function, especially when the patient is asked to name separately certain categories such as verbs and nouns, as they require attention and inhibition control [132]. Additionally, they ran a second study administering a semantic association task for verbs (KDT task) and for nouns (The Camel and Cactus Test CCT). They found a trend in the direction of a higher semantic impairment in verbs associations, confirming previous hypothesis. However, one limitation of this study — which could have an impact on the results — was that the action and object tasks had different psycholinguistics variables: the action naming task was easier than the object naming one, as the action pictures were more familiar and more frequent. This aspect highlight the importance of equalising the variables to achieve more reliable results [132]. In an fMRI study, Péran et al. (2009), investigated the brain regions involved in action and verb generation for the first time. Their results suggest that depending on the task demand, the PD deficits in frontal motor areas, affects semantic search. Although they did not find significative difference in the brain areas during objects or verbs generation, using a different statistical threshold they found more involvement of the prefrontal cortex, Broca's area and Anterior cingulate cortex (ACC) for action generation, suggesting a potential compensatory use of these areas given the frontal dysfunction in PD patients. When investigating the relationship between the motor deficit (frontal motor dysfunction in PD), and the different tasks, they found that regardless of the tasks, increases in the blood-oxygen-level-dependent (BOLD) activity in the supplementary motor area (SMA) and the left pre-central gyrus, was correlated with the severity of the motor deficit measured in participants using the motor part of the Unified Parkinson's Disease Rating Scale (UPDRS) [133]. This can be interpreted as a compensatory mechanisms which recruits other motor circuits to overcome the deficits in the striatal-frontal loop, consistent with findings of Bocanegra et al. of the involvement of the basal ganglia in action processing [127]. In general, there is consensus on the finding that PD patients display a deficit in action processing, the question is whether this impairment is caused by the involvement of cortical motor area in the semantic processing of actions, or whether this is due to a greater activation of frontal regions in processing verbs as a grammatical category. To investigate this,

Herrera et al. [134], ran a study where participants performed a picture naming task, where pictures had different degrees of motor content, using the International picture Naming Database (IPNP) [135]. Their hypothesis was that if PD patients' performance is affected by the degree of motion content in the picture, that would indicate a bigger involvement of brain area involved in motor control; instead, the lack of effect would support the grammatical hypothesis, where verbs as a grammatical category would rely more on frontal areas in the brain. Their results confirmed the hypothesis of a semantic action deficit in PD patients with a possible cause in the damage to the dopaminergic pathways mentioned previously, because they had significant lower scores in verbs with higher degree of motor content [134]. Similarly, a picture naming study assessing PD patients with and without cognitive impairment, showed sensitivity to the degree of motion content in the action pictures in patients without MCI. In this study patients with MCI showed a general impairment both for objects and action naming, but the performance was not affected by the degree of manipulability of the objects in patients without MCI, and it was instead affected by the degree of motion content in patient with no MCI. This study shows 2 different patterns: patients without MCI had a selective impairment in high motion action verbs, while patients with MCI had a more spread lexicon semantic deficit. This might be due to the fact that at the beginning the disease is characterised by fronto-striatal damage, that becomes then more pervasive affecting other areas such as the temporoparietal cortex [136]. The fact that objects and actions naming recruit different neuronal network is documented in literature, suggesting that left prefrontal cortex is recruiting during verb processing while the temporal lobe is more activated during nouns retrieval [137]. This difference could be useful in differential diagnosis, for example because action verbs seem to recruit frontal areas, neurodegenerative disorders with damage to those brain regions, can be distinguished thanks to this kind of linguistic pattern [138]. To test whether the deficit in action naming was due to the difference in the difficulty of the stimuli, some studies tested whether manipulability of the action depicted was a factor influencing performance, and the results show that in fact it is not a factor that influences performance in PD [133], while it was found in other motor disorders such as CBD [139]. These results support the theory of the existence of a strong correlation between action representation and verb processing. However, the deficit in object naming in PD

has been rarely explored, that is why in a recent study performance in a specific object naming task was investigated in PD patients, to assess the presence of anomia^x as patients complain about everyday word finding difficulties. This study investigates patients' language abilities through picture naming tasks and correlates the results with executive functions and working memory. A mild effect between groups in lower scores in naming was found, but there was no latency difference and a correlation with executive dysfunction was also found, but not major correlation with specific psycholinguistics variable [140]. More studies are needed to better understand the nature of these deficits, but most of the findings in literature seem to point to the direction of a *sui generis* impairment in action semantic processing, pointing to the importance of subcortical structures in language processing, also highlighting the existence of a dual pattern affecting differently patient without MCI and patients with MCI who show a wider spread impairment.

These findings are consistent with the neural exploitation hypothesis, a controversial theory suggesting that some neural mechanisms originally built for sensorimotor mechanisms, later on evolved to be used by the language systems while maintaining their original functions too [141]. The tasks mentioned above are the most used to assess any potential impairment in lexical retrieval abilities in PD, and especially ever since this dissociation between objects and verbs was detected, most research has focused on further exploring this aspect. A few studies have also tried to look at more spontaneous speech samples instead of lab based and single words or sentences by collecting longer speech samples, for example in the context of picture description. PD patients often complain about word finding difficulties from very early stages, but research has been limited on this matter especially because of the lack of quantitative objective measures. It is worth mentioning one study that stands out because of the attempt to quantify and automatise the analysis of spontaneous speech samples. To try and overcome this issue a study [71] tried to identify markers of cognitive decline in PD by analysing pauses in speech as an objective measure of Word Finding Difficulties (WFD). They recorded descriptions of the Cookie Theft picture [142] in PD patients without dementia and controls and found that PD patients had more pauses in utterances, fewer words per minute and lower well-formed sentences. These findings could constitute early markers in PD patients without

^x Anomia refers to a form of aphasia where patients have difficulties in recalling names of everyday objects.

dementia and this protocol could be applied to other neurodegenerative disorders [71].

Receptive language

Despite being less studied than language production there is evidence that language comprehension is also impaired. PD patients showed impairment in an experiment with polysemous words where participants had to select words or non-words. The results demonstrated a prolonged priming effect^{xi} in PD patients, supporting the presence of a potential impairment in inhibitory semantic mechanisms [143]. Comprehension of syntactically complex sentences also seems to be impaired in PD patients. In an experiment to investigate whether the causes for sentence comprehension deficits in PD patients lay in executive functions deficits, participants were asked to judge whether a transitive sentence entails an intransitive one. Particularly, it is believed that working memory is impaired and that complex sentence comprehension makes great demands on the central executive components of the working memory. A set of specific high demanding verbs were chosen, assuming higher demand on executive functions and different mapping in the brain. The results showed that PD patients were impaired in these kind of sentences supporting the hypothesis that executive function impairment might be the cause of their deficits [144]. Other studies were able to match impairment in sentence comprehension with memory and attention by proposing increasing grammatically complex sentences to participants, and finding that the difficulties of patients became more evident as the complexity increased [145] [146]. On the other hand, there is also evidence supporting the embodied cognitive theory, which suggests that certain motor sensory circuits and cortical-subcortical systems, such as the basal ganglia network, are involved in language processing. In this framework, semantic memory is grounded in those sensory motor systems, as action verbs comprehension is correlated with motor impairment rather than executive dysfunctions. Additionally, PD patients have more difficulties in understanding sentences with higher action content than low action content, where non MCI patients showed action meaning deficits not correlated to their executive functions and general cognitive abilities [147]. More studies are necessary to understand the nature of this deficit.

^{xi} Priming effect refers to the phenomenon where exposure to a certain stimulus might influence response to a subsequent stimulus.

Alzheimer's Disease

AD is the first leading cause of dementia worldwide and is currently becoming one of the most prevalent diseases of this century [148]. AD is a neurodegenerative disorder affecting many different areas of the cerebral cortex and hippocampus. At the first stages of the disease it affects areas that involve the temporal and frontal lobe to spread further in other areas at a different pace that varies across individuals [149]. As in many other unremitting neurodegenerative disorders the main goal of research is early diagnosis, and because of the complexity of the mechanisms characterising this disease, it is difficult to build effective diagnostic instruments able to detect AD at an early stage. In recent years language is becoming a simple but effective procedure to try and tackle the disease potentially at a preclinical stage. Particularly certain aspects such as fluency and verbal ability seem to have a high level of accuracy in distinguishing between healthy controls and AD [150].

While one of the most used techniques for investigating language abilities in PD is the use of Picture naming and verbal fluency tasks, in Alzheimer's Disease research has mostly focused on tasks that would capture connected speech like picture description tasks, more similar to real life abilities. Nevertheless, some studies have tried to identify specific pattern in language AD picture naming, especially to differentiate their performance from PD patients at the earliest stages.

Language production

A large number of studies argued that object naming is more impaired in AD patients compared to action naming, because of the locus of the brain area affected by the disease: AD patients at the early stages of the disease are more impacted in the temporal lobe areas, where object semantics are processed, while actions are usually processed in frontal areas as mentioned in the section exploring literature on PD. Studies directly comparing object and action naming between AD patients and controls, found a bigger impairment in object compared to actions [151-153] when using tests like the Boston Naming Test and the Action Naming Test [153], or in a single case study where a 79 year old female patient was administered the Graded Naming Test and the Oldfield Picture Naming Test, resulting in with very low scores [152]. Other researchers did not find the same pattern on administration of similar tasks, such as a study where a group of Italian AD patients were compared with a control group and

FTD patients which found a greater impairment in naming in both dementia groups compared to controls, but the FTD group had a bigger dissociation between action and object naming, supporting again the hypothesis of action naming being rooted in frontal areas [154]. Similarly, a study on Chinese patients, as reported in Materson, Druks et al. [155], found no differences in object and action naming. The same study did not confirm the hypothesis of preservation of verbs compared to nouns in picture naming, as they found AD patients and the control group to be faster on naming objects compared to actions and even the accuracy levels were higher in objects for both groups. However, one limitation of this study is that they matched the stimuli only for one psycholinguistic variable, such as age of acquisition, which as a result did not account for other variables known to be important in these kinds of studies, such as frequency for example. An interesting finding of their study was that object and action naming elicited different kinds of errors, suggesting that the two categories pose different cognitive demands when performing the task [155]. A few studies directly compared performances of PD and AD patients; a study by Pignatti et al. [156] did not find any significative difference between the groups in picture naming, but a higher object-verb ratio was identified in PD patients compared to AD. They also analysed a spontaneous speech sample and did not find any significative difference in verb and noun production, although AD patients produced fewer nouns than the PD group. In general the picture naming confirmed that PD patients have more issues in producing verbs, while in spontaneous speech samples the pattern seems to be quite opposite, with AD patients having in general a less rich verb pool in storage [156]. This study has the merit of putting together analysis of a standardised and constrained task such as the picture naming one and a spontaneous speech sample, and both are important because each one can inform on different aspects about the nature of the impairment. Picture naming seems to elicit more consistent results in PD than in AD, and this can be caused by several reasons: the stage of cognitive deterioration, the task administered, the language used, and the psycholinguistic variables considered. However, using different types of tasks to investigate language abilities can reduce the variability in different research and improve test-retest reliability. Picture description tasks seem to be the more consistent, hence more suitable to detect potential linguistic pathological patterns in AD.

In a picture description task in Japanese speakers, researchers tried to compare performances on three groups of subjects: a group of individuals with amnesic Mild Cognitive Impairment (MCI), a group with mild AD and a healthy control group. They were tested on the Communicative Abilities in Daily Living (CADL), on a confrontation naming test (comparing actions vs objects), and on a picture description task using The Cookie Theft Picture Description test [157]. The results showed poorer performances on the CADL and on the naming test only in the mild AD group. In the picture description task the syntactic analysis generated no differences. The semantic analysis showed differences even between the MCI group and the control group. This result is quite important, because it can show patterns that can be discovered early on in the disease. The authors of this study explained that the picture description task is probably complex enough to require planning, maintaining activation of the basic language operations at different levels such as the lexical, semantic, syntactic, phonological and at the monitoring level. It is still quite unclear what kind of brain areas and networks are recruited during this kind of task because it depends on many different aspects that are task specific, on the level of cognitive impairment and on individual differences. However, recent studies seem to have identified some areas that are recruited during complex, time-constrained attention based language tasks: the left anterior and bilateral posterior perisylvian regions, as well as the prefrontal cortex and contralateral posterior neocerebellum [157]. Another study investigated the network involved in picture description tasks by pairing administration of a picture description task and N-isopropyl-p- [123I] iodoamphetamine and single photon emission CT to measure regional cerebral blood flow (CBF) [158]. In this study verbal ability of AD patients and controls was analysed for: (a) Informativeness, as AD patients often display non-informative language features (b) the number of constituents produced for the depicted picture, (c) the amount of relevant and irrelevant description, (d) efficiency, (e) appropriateness, (f) sentence structure [158]. Only (a), the amount of information produced and (d), efficiency of description resulted in significant differences compared to the control group as their performance was less informative and efficient. The investigation of CBF using SPECT examination showed activation of the left-thalamus and the occipital lobe, areas related to verbal ability. The regional areas identified correspond to the visual cortex, an area that is clearly needed for this kind of task. Interestingly,

left thalamus cortical connections indicated this area as probably involved in verbal description ability [158]. Analysis of connected language seems to be particularly appropriate to detect changes early in AD. Connected speech is the study of self-generated discourse often elicited by a visual prompt, like in picture description tasks [159]. The success of this task is related to the easy administration procedure which at the same time is relatively low demanding for participants and investigates the ability of storing different kinds of linguistic information in working memory to be used at the appropriate time, and it mimics more closely real-world language production. Additionally, because language in AD dementia has been described as empty, non-informative, with a strong use of circumlocutions [159], this kind of task that requires specific information to be conveyed is a perfect tool to assess language in these cases. In a recent review Mueller et al. [159] provide an overview of the most important findings in picture description tasks in MCI and AD, and we believe it is worth summarising here the highlights of their results as it provides an interesting linguistic profile for AD that could be helpful to be compared with other disorders and in clinical settings. During picture description the visual prompt is visible throughout the task, hence participants rely less on episodic and autobiographical memory. This is important especially in AD, where the main deficit seems to be related to semantic language, elicited by picture description. One of the most used in literature is “The Cookie Theft” picture, which is designed to elicit aspects that are relative to people, time, actions, and the main vocabulary pertains to words usually learnt early in infancy [142]. The linguistic profile that emerges from this review is very interesting, as they demonstrate that the main patterns are identifiable at a later stage, but some signs can be found even earlier on at the MCI stage. Some theories suggests that language changes are due to “suboptimal neurocognitive development” [159] putting the subjects at a higher risk of developing neurodegeneration. This idea has been developed following the investigation of an important measure applied to connected speech, such as propositional idea density (P-density), a measure that is obtained by counting the number of content words relative to the numbers of total words in a sentence. A famous retrospective study was able to identify low density in a sample of early life written language of a group of nuns, and the P-density measure was able to predict cognitive decline later in life [160]. These fascinating studies have the merit of pointing to a process that is impacted before the clinical diagnosis is

possible, but the real challenge is to be able to detect it before the development of symptoms. Additionally, written language is very different from spoken language, and it engage a lot of different psycholinguistic variables and processes, as well as different neural networks. Therefore, it is important to understand how to identify measures that help detect similar changes in spoken language. The main findings of the review outlined a semantic deficit identifiable at different stage, starting from pre-symptomatic, across different stages of MCI and then mild to severe AD. Additionally syntax seems to be also impaired starting from the MCI stages [159]. Despite what is generally believed, picture description can distinguish between AD and controls, but it usually does not correlate with standardised tests such as picture naming or verbal fluency tasks, reinforcing the idea of picture description being the more suitable to identify pathological patterns in this population.

Finally, the authors of the review suggest implementing longitudinal studies with picture description tasks that are now very few and they point out the importance of studying syntax as it is possible to detect changes in that aspect quite early in the disease. Furthermore, they address the need for more automatic and standardised measures of picture description, not only to reduce the time of manual analysis, but also to make it more reliable and reproducible [159].

Receptive language

As seen in PD one of the most studied aspects in language comprehension is sentence comprehension. Several studies focus on whether syntax as well as semantics are both impaired in AD. Some studies did not find impairments in syntax leading to the idea that the latter is in fact not impaired in AD patients, supporting the hypothesis that language is modular and that semantic and syntax have distinct processing modules in the brain [161]. Other studies instead, found that syntax and sentence comprehension may both be impaired in AD using sentence-picture matching tasks, where AD patients performed significantly worse than controls. However, they also found a compensative role played by morphology in certain tasks where sentences richer in morphemes were processed more easily than others [162]. Semantic deficits have also been found in AD, particularly in word comprehension as expressed in studies investigating semantic priming^{xii} effects. Interestingly, greater priming effects were found in

^{xii} Semantic priming is a linguistic concept referring to the effects of facilitation that a semantic related word might have on retrieving a subsequent word 163. Hoedemaker, R.S. and P.C. Gordon, *The onset and time course of*

AD patients compared to controls, a result consistent with the idea that AD patients have degraded semantic network activation, hence fewer intact connection nodes, which causes activation to spread more quickly ^{xiii} [164]. A possible explanation for AD linguistic deficits could be attributed to deficits in working memory ability which is known to be fundamental in a lot of linguistic processes [161]. Whether this is due to a capacity reduction, limiting the amount of online information that AD patients can temporarily store in the working memory to be then used, or if it is due to speed of processing, it is still unknown. Another possible explanation could lie in the difficulties of post linguistic interpretation, posing demands on different cognitive abilities that being also impaired causes difficulties in AD comprehension of complex sentence [161].

Corticobasal Syndrome (CBS) and Progressive supranuclear palsy (PSPs)

Corticobasal Syndrome (CBS) is a neurodegenerative disorder characterised by a combination of motor symptoms, such as tremor, dystonia and some symptoms related to cortical functions, such as alien limb syndrome and apraxia [165]. The affected brain regions include areas of the frontal and parietal region, and the basal ganglia. Many cognitive functions are impaired, for example language, working memory, executive functions, social cognition and visuo-spatial awareness [165]. Progressive supranuclear palsy (PSP) is a complex progressive neurodegenerative disease; amongst the main features, patients can display dysarthria, dystonia, supranuclear ophthalmoplegia, pseudobulbar palsy and dementia. The brain areas affected are mainly located in the midbrain and in the frontal areas, and demyelination in the basal ganglia, brain stem and cerebellum have been also identified [165]. Among the clinical criteria for diagnosis, speech and language are also considered an important cognitive function affected.

Language production

Fluency is usually affected in both disorders; therefore, verbal fluency tasks are often used to investigate language; particularly first letter fluency tasks show significant deficits [166, 167], which could be explained by the fact that neuroimaging findings show activation in frontal regions with this kind of task. Another distinctive feature of these patients is that they produce low frequency words compared to Alzheimer's patients that usually tend to produce more high

semantic priming during rapid recognition of visual words. J Exp Psychol Hum Percept Perform, 2017. **43**(5): p. 881-902..

^{xiii} For a further explanation of lexical access theories see section 2.2.1 of this chapter.

frequency words [168, 169]. The reason behind this impairment in fluency is yet to be explained, it might lie behind executive function deficits, working memory or attention, or reduced speed of processing which has been documented in both disorders [170, 171]. Picture naming has also been used to investigate language in CBS and PSP, although only mild impairment or no impairment has been found in studies investigating naming in PSPs [170, 172], while in CBS it seems to be more consistently impaired, although only mild difficulties have been reported in the literature [173]. There is a lack of reports of the type of errors made during naming tasks in CBS and PSP; therefore more studies are needed to determine the nature of the impairment found as Person et al. reported in their review [165]. In both PSP and CBS there seem to be a clear impairment for action naming compared to objects [174], supporting the hypothesis of action naming being represented and processed in frontoparietal-subcortical networks [165].

Receptive language

In CBS patients there is evidence in the literature showing deficits in single word comprehension [175], although the deficit was not found in every study [176]. Sentence comprehension has been found to be relatively unimpacted while grammatical judgement was impaired in the same study by Cotelli et al. [53], leading to the idea of a potential specific syntactic deficit in CBS.

Regarding language comprehension in PSP, patients seem to be more affected in sentence comprehension than word comprehension. Specifically PSP patients were significantly impaired in tasks requiring initiation and search strategies, but their performance was relatively spared when it comes to simple word comprehension [177]. However, both action verb single word comprehension and sentence comprehension were significantly impaired in a study investigating performance on different tasks [178]. Performance of PSP patients was also impaired in tests requiring semantic association, where however a great deal of executive function ability is required to perform those tasks and no visual impairment which might affect performance [179].

Frontotemporal Dementia (FTD) and Primary Progressive Aphasia (PPA).

Frontotemporal dementia (FTD) is a cluster term used to identify different variants of a group of early-onset neurodegenerative disorders with different characteristics encompassing behavioural aspects and language deficits. The variants where language deficits are prominent and represent the main clinical

features are grouped into the umbrella term of Primary Progressive Aphasia (PPA), while the variant characterised by primarily evident behavioural and emotional changes is called behavioural variant frontotemporal dementia (bvFTD). Interestingly, the latter is also characterised by language deficits [180].

Language production and reception

Naming abilities are usually frequently impaired in bvFTD as seen in the frequent use of the Boston Naming Test and seems to be particularly impaired in action naming. Word comprehension also has been shown to be impaired while sentence comprehension is most frequently preserved in general, but impaired when using more complex sentences. Connected speech studies showed the presence of disfluencies such as more frequent silence pauses, while at a discourse level difficulties in discourse organisation and accurately reporting events were found [181]. Primary Progressive aphasia (PPA) refers to the condition of patients characterised by language deficits which constituted the main clinical feature that progressively deteriorates [182]. Currently, PPA is taxonomically divided in three subgroups with different language deficits:

- The nonfluent/agrammatic variant characterised by language production deficits such as speech fluency being progressively affected with demand posed on naming actions compared to objects, and grammatical errors.
- The semantic variant (svPPA) does not affect speech fluency but rather semantic memory hence naming abilities and word comprehension.
- The logogenic/phonological variant (L/phvPPA) is characterised by word finding difficulties expressed in disfluencies such as long pauses, slow speech and anomia, as well as sentence comprehension deficits [182].

In general naming difficulties is one of the first clinical features affected. Anomia is present in the different variants and can result from the semantic deficit in svPPA, or on post semantic interpretation difficulty at the phonological level, for example in L/phvPPA as seen in other neurodegenerative disorders [182].

Fragile X-associated tremor/ataxia syndrome (FXTAS)

FXPCs can develop a neurodegenerative disorder characterised by tremor, ataxia, Parkinsonism, and cognitive and behavioural changes, called Fragile X-associated tremor/ataxia syndrome. Symptoms usually manifest in individuals in their 60s and MRI findings report brain atrophy, white matter changes,

particularly in the middle cerebellar peduncles and parts of the corpus callosum. Brain atrophy has been identified in the cerebrum and cerebellum, in the dorsomedial frontal-parietal regions, insula, medial temporal regions, thalamus and striatum. Additionally ventricular enlargement and thinning of the corpus callosum occur at the latest stages of the disease, although these changes are less severe in females [183]. Memory, attention and executive functions can be affected, and about 50% of male FXPCs can develop the neurodegenerative conditions, while females have less chance to develop dementia due to the protective function of the second X-chromosome that does not carry the mutation [183].

Despite the huge within-syndrome variability, most individuals with FXTAS have spared speech and language functions, as comparative studies with other disorders such as AD have demonstrated [184]. Some individuals can develop dysarthria due to the affected performance of the cerebellum, and language measures that are more directly connected to the executive functions, such as verbal fluency tasks, seem to be compromised. Some deficits are also found in females with FXTAS where verbal learning and executive functions in females were affected compared to controls [185]. One of the greatest challenges in FXTAS is to correctly diagnose the disease and distinguish symptoms and features from other neurodegenerative disorders such as PD. Only family history usually leads to genetic testing and the correct diagnosis, and it is still unknown why some individuals with

1.5 Molecular measures: plasma-based proteins as a candidate biomarker

One of the goals of our molecular analysis is based on the important issue of finding minor invasive ways to collect fluid biomarkers. Looking for potential biomarkers detectable both in CSF and in other fluids such as blood, urine, saliva etc, is a fundamental goal of drug discovery research, as it is less invasive, cost effective and overall easier to carry out both for the researcher and the patients [186]. The molecular analysis in this project will concern measurement of plasma-based proteins alteration in FXS and FXPCs, changes which have been shown to be reflective of similar alterations happening in the brain where they are indicators of an important cytoskeletal mechanism, vital for synaptic plasticity: Microtubule dynamics [187]. These aspects will be discussed in

Chapter 3. Here, we provide a brief introductory overview of the microtubular structure and its function, which will be elaborated more in detail later, as well as a preliminary discussion of the rationale for measuring proteins associated with microtubule dynamics in plasma.

As mentioned in the pathophysiology of FXS in section 1.2.2, the full mutation causes silencing of the *FMR1* gene and loss of FMRP protein production. Many functions of FMRP are still unknown, but it is known that it is an mRNA-binding proteins that regulates transcription of mRNAs, and it is involved in encoding other proteins which regulates dendritic spine expression [188]. Therefore it is believed to be involved in microtubule presynaptic structure and function [189]. Microtubules are a very important structure of cellular organisation. They represent a fundamental component of the cytoskeleton, and are essential organelles involved in different cellular processes, such as transport of vesicles, cell division, morphogenesis and organisation [190]. They are formed by the Alpha and Beta tubulin heterodimers which polymerise into protofilaments which assemble to form a tubular structure called microtubule. The outer diameter of each microtubule is 25nm and it is polarised to a “plus” end which expose the Beta subunit and a “minus” end exposing the alpha subunit.

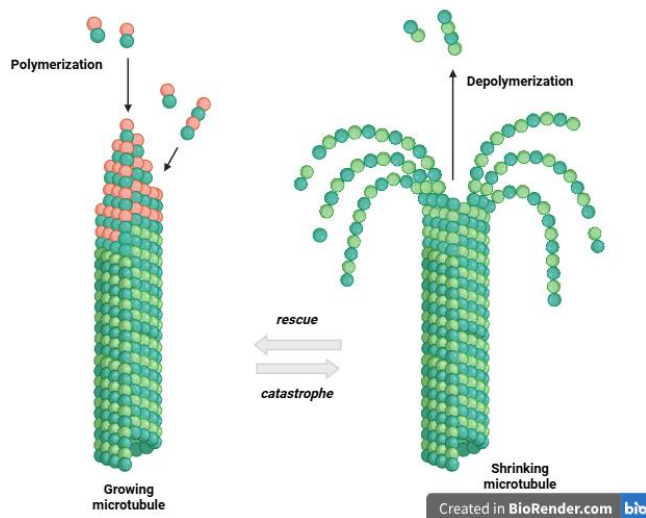
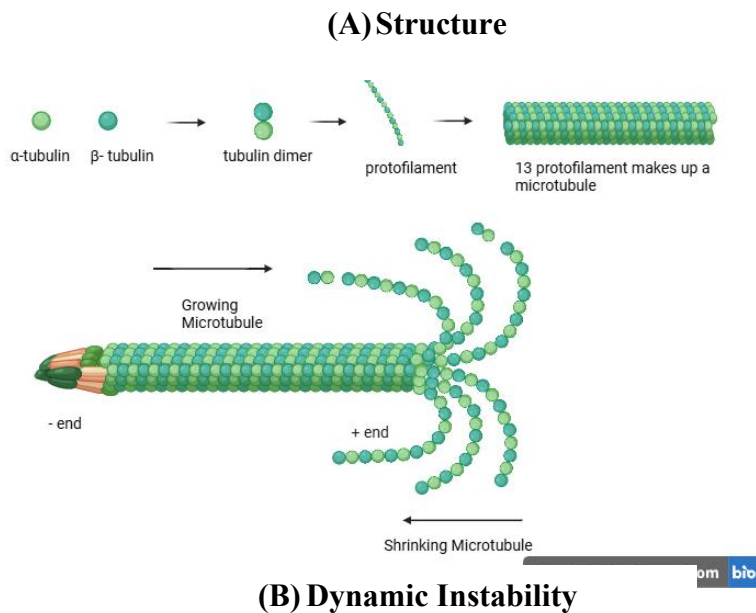


Figure 1: Microtubules structure and dynamic.

(A) The alpha and beta tubulin dimers polymerise into protofilaments which assemble to form a tubular structure called microtubule. Each microtubule is polarised to a “plus” end which expose the Beta subunit and a “minus” end exposing the alpha subunit. (B) Dynamic instability refers to the property of the plus end to alternate moments of prolonged assembly and rapid disassembly. Catastrophe is the transition from slow assembly to rapid disassembly, rescue is the process from rapid disassembly to slow assembly [190]. Created in <https://BioRender.com>

A very important phase of microtubule assembly is called steady-state, and it consists of a balance between a majority of slowly growing microtubules and a minority of rapidly shrinking microtubules. This phase has been studied since the 1980s, to measure the growth rate of microtubules [191]. It represents the last and more dynamic stage of assembly where microtubules are always out of equilibrium.

Two mechanisms are responsible for this phase:

- **Treadmilling:** which is linked to the plus end, where microtubules continuously incorporate new dimers, while the minus end continuously loses dimers.
- **Dynamic instability:** this represents the property of the plus end to alternate periods of prolonged assembly and rapid disassembly [190].

We will focus our attention on the second mechanism: the dynamic instability or microtubule dynamics. Dynamic microtubules are fundamental in neuronal plasticity as they play an important part in the remodelling and extensions of axons and dendrites. Dynamic instability gives microtubules the possibility of creating the intraneuronal space and provide the pathway to the new synaptic formation. The highly dynamic microtubule it is involved in many different processes during early embryonic growth, for examples cell division, intracellular trafficking and to provide a guide to synapse formation. Therefore, the support of microtubules is fundamental both in neuronal connections and then, once the connections are established to provide support and maintenance of these connection during development [192]. Microtubules undergo several post-translational modifications (PTMs) which regulate the formation and maintenance of the polarised morphology of neurons. Particularly, acetylation and tyrosination play an important role as the expression of specific alpha-tubulin isoforms such as tyrosinated alpha-tubulin (Tyr-Tub) and acetylated alpha-tubulin (Acet- Tub) which are both markers of the microtubule dynamics. Several studies have shown that alteration and dysregulation of tubulin isoforms are linked to a broad spectrum of pathologies, including neuropsychiatric [193], neurodegenerative [194], and neurodevelopmental disorders [195]. In the hippocampus of animal models of depression increased levels of Acet-tub have been found and they possibly indicate decrease microtubule dynamics and possible disfunction in microtubules [190]. Interestingly, alpha-tubulin PTMs are also involved in peripheral cells activation and total expression of alpha tubulin has been shown to be detectable not only in the brain but also in rat plasma [196]. Previous studies have shown changes in hippocampal α -tubulin PTMs in rodent models of depression indicative of reduced microtubule dynamics and therefore reduced neuronal plasticity [193]. Importantly, it has

been shown that healthy volunteers treated with ketamine have altered plasma α -tubulin PTMs which may be reflective of changes in the brain [187]. Moreover, recent data obtained by Ulysses Neuroscience show that in patients with major depression there is a significant correlation between plasma levels of α -tubulin PTMs and severity of their symptoms (manuscript in preparation). Furthermore, growing evidence suggest that alterations in microtubule dynamics are a key factor in the pathogenesis on X-linked neurodevelopmental disorders such as FXS and CDD [195]. Recent studies at Ulysses Neuroscience Ltd. have shown that *Fmr1* knock-out (KO) mice, a model of FXS, have increased Acet-Tub/TOT-Tub and decreased Tyr-Tub/Glu-Tub in the hippocampus compared to wild type (WT) mice (manuscript in preparation). Interestingly, the increase in hippocampal Acet-Tub/TOT-Tub was also observed by the same team in *Cdkl5*-KO mice, model of CDD, a rare neurodevelopmental disorder resulting in severe early-onset seizures, intellectual disability, motor, and social impairments. The increase in Acet-Tub/TOT-Tub, was paralleled by increased plasma Acet-Tub/TOT-Tub (unpublished data). Furthermore, plasma samples of CDD patients from Ireland, Italy, and the US were analysed by the Ulysses Neuroscience Ltd. team. They analysed Acet-Tub/Tot-Tub and Tyr/Glu-Tub by Infrared Western Blotting using the Odyssey CLx imaging system (Li-Cor), a technique that will be further discussed in Chapter 3. Increased in Acet-Tub/Tot-Tub was found in human plasma like that seen in the mouse model, confirming the hypothesis that it is possible to observe in plasma changes in microtubule dynamics that could be reflective of changes happening in the brain Figure 2. Additionally, a range of inflammatory markers were analysed^{xiv}, BDNF^{xv}, and NfL through electrochemiluminescence ELISA (MSD), a multiplex assay able to quantify a arrange of multiple targets at the same time; we will also further discuss this technique in Chapter 3. Acet/Tot-Tub, NfL and IL-10 were overexpressed in plasma samples of patients, potentially indicating respectively dysregulation in proteins related to microtubule dynamics in the brain (Acet/Tot-Tub), in a known marker of axonal damage (NfL), and in a potent anti-inflammatory marker, such as Interleukin-10

^{xiv}Inflammatory markers are indicators of body inflammation and are analysed here to check for potential dysregulation in immune response. Here we report results of a cytokine known for its potent anti-inflammatory effects, interleukin-10 (IL-10)

^{xv} Brain Derived Neurotrophic Factor (BDNF) is crucial for neuronal survival and differentiation playing a fundamental role in synaptic plasticity. 197. Miranda, M., et al., *Brain-Derived Neurotrophic Factor: A Key Molecule for Memory in the Healthy and the Pathological Brain*. Front Cell Neurosci, 2019. **13**: p. 363.

(IL-10), whose upregulation has already been described in CDD patients [198]
Figure 2.

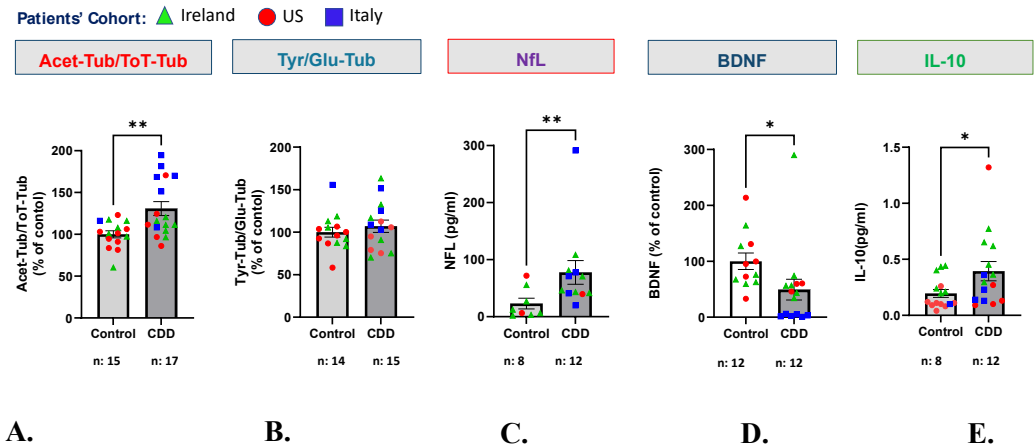


Figure 2: Plasma Acet/Tot-Tub, NfL and IL-10 are overexpressed in CDD patients and BDNF is downregulated.

A. Acet/Tot-Tub ratio is significantly increased in the plasma samples of CDD patients. B. Tyr/Glu-Tub ratio is not significantly dysregulated in plasma sample of CDD patients. C. NfL is significantly increased in plasma samples of CDD patients. D. BDNF is significantly decreased in plasma samples of CDD patients. E. Interleukin-10 is significantly increased in plasma samples of CDD patients. Data expressed as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. controls.

To the best of our knowledge, no one has investigated potential alteration of these proteins in FXS and in FXPCs at the same time, and given the potential role played by FMRP in synaptic plasticity, we believe it is worth carrying out this molecular analysis in patients with FXS and in FXPCs, with the goal of gaining a better understanding of the role played by the different kind of mutations on the *FMR1* gene.

The original design for this project was to develop an integrative complex candidate biomarker, combining language assessment and molecular analysis, in both FXS and FXPCs. However, our PPI approach led us to a change of plans. We strongly believe in patient engagement from the design to the results dissemination of a research study. Therefore, following discussions, meetings and focus groups with the Irish Fragile X Society, we changed our plans based on concerns about the feasibility of the study with children with FXS, and we agreed to run a study with FXPCs, mostly parents of children with FXS, with the future goal of expanding the study on FXS children. Therefore, in this manuscript we will present the results of the new design developed involving the patient community, which includes two studies on FXPCs to develop a potential composite biomarker (in chapter 2 and 3), and the results of a pilot retrospective study on FXS children (Chapter 3), where analysis of the plasma-based proteins

was carried out, to build the foundation for further studies on FXS. In the next section we will describe more in details the patient focused approach, and the objectives of this research project.

1.6 The importance of patient centricity and the patient focused drug discovery (PFDD) recommendation from the FDA. 5000 words

The patient focused drug development Glossary defines the “Patient-focused drug development (PFDD) —also referred to as patient-focused medical product development— as *“A systematic approach to help ensure that patients’ experiences, perspectives, needs, and priorities are captured and meaningfully incorporated into the development and evaluation of medical products throughout the medical product life cycle”*[199]. Despite the importance of patients in drug discovery and development, it is only recently that regulatory boards such as the Food and Drugs Administration have started to regulate the involvement of patients in the research process. In 2012 the FDA established the PFDD to enhance the experience of those living with specific condition as they are *“uniquely positioned to inform the understanding of therapeutic context for drug development”* [199]. The initiative consists in holding a series of meeting with the goal of hearing the patient voices to obtain a sense of what is like to live day to day with a disorder: particularly focusing on the symptoms they experience, their opinion on the current treatment approaches, and how patient wants to be engaged in the drug development process. From 2012 the FDA has held specific meetings for different conditions, and the reports are publicly available. The FXS meeting was held in March 2021 with the National Fragile X Foundation and researchers, clinicians, and patients gathered to better understand how to improve the patients’ experience. The report summarised some of the needs presented by patients, and the main ones concern the need to treat the disorder as a whole instead of just the symptoms, the need for better outcome measures, particularly for composite outcome measures to monitor improvement on different aspects, the need for treatment that would improve cognition hence the quality of life, and the need to involve parents and caregivers in the research design process at all stages [31]. Furthermore, the need to involve parents and caregivers is certainly fundamental for any kind of neurodevelopmental disorder, however, becomes even more important for

parents of children with FXS, as often they are living themselves with a series of conditions connected to the *FMR1* gene mutations. This point is particularly important, not only because of the expertise that only patients and caregivers could offer on the disorder they live with, but also because they can advise better than anybody else on the feasibility of a particular study design of assessment. If we go back to the question “What makes a biomarker a good candidate?” asked in section 1.3. we can now add to reproducibility and quality a third point: “feasibility”.

As our empirical experience in designing this study showed, it is only with the expertise of those dealing with a condition day to day that one can assess the feasibility of a study design, therefore it is vital to involve patients, family members and caregivers in any research project, including clinical trials.

1.6.1 From the Irish community to the international community

It is not always easy to actively practice patient engagement when conceiving the idea of a study: as a matter of fact, often the available resources in terms of funding and time are not sufficient, and gathering a community, especially of a rare disease requires a lot of effort, especially in terms of providing the necessary support to the community and gaining patient’s and families’ trust. Nevertheless, we strongly believe that the overall goal of research should be improving the quality of life of those involved, consequently we strive to achieve a real involvement of the community into the research design, to empower patient’s families and at the same time gain insights on what is needed, what kind of approach is necessary and what needs to be changed.

To achieve this, we took several steps:

- First, we met with representatives of the Irish Fragile X Society to propose the first idea of the project, and after a few discussions and meetings the study involving the children’s language assessment did not appear feasible. Therefore, in agreement with the community, we decided to start a project investigating the possibility of developing a composite measure for FXPCs, often neglected by research.
- Secondly, we kept involving the Irish community in the development of the research study, inviting them to all the several awareness days and

family days organised by Ulysses Neuroscience Ltd., for which patient-centricity is one of the core values of the company.

- Additionally, we expanded our perspective by starting to reach out to the international community, and thanks to the FRAXA research foundation, we were able not only to engage with different clinicians working on FXS all over the world, but we were able to virtually meet with a US family affected both by FXS and FXPAC and listen to their experience, which was recorded in a podcast in July 2021.^{xvi}
- Lastly, in October 2022, in occasion of the European Fragile X Day we organised a FXS Family Awareness Day to present the project design, focused on FXPCs. We then started recruiting participants with the help of the Irish Fragile X Society for an in-person study, that combined assessment of language abilities and analysis of plasma-based proteins.

After several months of active recruitment, the number of participants was still quite small, therefore we decided to expand the linguistic part of the study online and involve the international community. Thanks to the endorsement of the Irish Fragile X Society, we reached out to several associations and foundations around the world, including Fragile X International (FRAXI), the UK Fragile X society and the American National Fragile X Foundation. The latter led us to connect with the International Fragile X Premutation Registry [33]. This initiative was launched in 2020, initially able to include only US residents, it was then expanded internationally in 2021 thanks to the UC Davis MIND Institute. The Registry has the purpose of helping connect researchers and patients, by creating a group of Premutation Carriers all over the world, who are willing to participate in future research and who are informed of the new studies being developed. Each research study goes through an evaluation process and is assessed by a board of experts before being submitted to potential future participants, it is only after full approval by the board that the recruitment can start. We submitted our application to the registry and thanks to their approval we were able to reach an international community and involve online participants based in many different

^{xvi} Ulysses Neuro Podcast is a series of podcasts about giving voice to the patient community around the world. Each episode collects the stories of different people living with different conditions, and on July 2021, in occasion of the American International Fragile X Day, in collaboration with FRAXA, we interviewed a mother who is a FXPCs and her daughter living with Fragile X Syndrome. The episode could be found on the main streaming platforms.
<https://open.spotify.com/episode/4BEWhMYrYofCEWB9NgHmMy?si=44518851cb18464c>

countries, including the United States, South Africa, Ireland, the UK, Germany, the Netherlands, Australia, and New Zealand. This international approach and the decision of carrying out the study through zoom calls with participants, not only helped us in reaching a larger sample size needed to carry out an appropriate analysis of the data, but most of all enriched the quality of the recruitment and data collection process, as we have been able to listen to the experiences of so many different people, to enter for a brief moment in the family life of so many different communities and cultures, with just as many stories to share. Additionally, the methodology of this study is consistent with new FDA guidelines and recommendations for decentralised clinical trials (DCTs), where trial related activities take place in locations other than the traditional sites, such as homes of trial participants for example. This new approach is fundamental because it has been shown to reduce drop outs rate, to ease participation in many aspects and to make it easier to involve patients from the very early stages of the trials design [200]. In conclusion, by including patients and parents from the beginning we were able not only to listen to the patient voice but also to give the community an opportunity to be at the centre of the research and design process. FXPCs will develop the neurodegenerative disorder while other will not. As in many other neurodegenerative disorders one of the main goals is to determine whether signs of cognitive decline can be identified early on. Language once again could be an asset in this kind of investigation; although most individuals with FXTAS will preserve their language abilities, some findings in literature have shown that FXPCs can show signs of cognitive alteration in linguistic features that can help distinguish from controls. As seen in the previous sections, a few studies found measures that can potentially point to individuals more at risk of developing cognitive decline. Most of these measures were concomitant with signs of disfluent speech that would involve disturbances in the flow of information such as repetitions, self-corrections etc [7]. All the studies that investigated language abilities in FXPCs so far were able to find more disfluencies compared to control participants, a clear effect of age, word retrieval difficulties and the use of shorter and simpler sentences compared to controls. They were also able to find a correlation with the number of CGG repeats, but not all of them were able to correlate the disfluencies to an executive function deficit, as disfluent speech is usually associated with difficulties in planning and a general deficit in executive functions [107] [8] [108]. This latter finding

suggests that the cause of the higher presence of disfluencies is still unclear, and might also be related to compromised functions of more language specific processes, such as lexical access, that involve areas such as the inferior frontal gyrus, whose involvement in language functions has been connected to the cerebellum, particularly related to correct decision making in language functions [201]. Both the cerebellum and the basal ganglia seem to be connected to a procedural system related to the correct representation of words [201]. This is particularly relevant for the purposes of this study as we will further explore later in the chapter, as accelerated volume decrease in the cerebellum and whole brain have been identified in FXPCs too [202]. Additionally, one of our main aims is to explore the existence of potential differences between FXPCs and controls, both in tasks tapping directly into the lexical access process and in a picture description task, which could potentially elicit disfluencies as previous findings in literature have demonstrated. We believe that a potential correlation between performances in these different tasks could advance research on the potential explanation of the disfluent language patterns found in FXPCs. One of the limitations in the reviewed literature on language and FXPCs is that the studies have usually a higher number of females compared to males because the premutation is more common in females (1 in 200 women and 1 in 400 men) [183], and because of this gender difference prevalence in the development of FXTAS and dementia in this population, more studies including male FXPCs are needed. Only one recent study included both symptomatic males and females, making it more feasible to be used as outcome measures for clinical trials [109]. To conclude this review section on language in neurodegeneration, the findings reported highlight that language is affected not only in widely recognised language-impacted neurodegenerative disorders like PPA or AD, but even other disorders like PD which was considered spared can show impaired language features. Studying these features can be a very useful tool to distinguish between controls and individuals showing pathological signs, and for differential diagnosis. Additionally standardised measures are required to improve generalisation and reduce the subjectivity of the investigator, as well as studies in languages other than English, as most of the above-mentioned research were focused on English. Of the world's 7000 languages, studies on language in neurodegeneration span only across about 20 languages. This finding would not be problematic if languages shared all universal features, as some theories

suggested in the past; but thanks to technological advances in neurolinguistic studies, we now know that typologically different languages could engage different brain regions, and for example they can entail differences in word categories such as nouns versus verbs, which is widely explored in neurodegeneration but might not be evident in tonal languages [75].

Chapter 2: Linguistic experiment 1: biomarkers in Fragile X Premutation Carriers: language markers of cognitive alterations.

2.1 Introduction

In this chapter, we will describe the experiment focused on assessing language measures and we will address the first two research questions (a) Is it possible to identify potential differences between FXPCs and a control group through language assessment that could indicate prodromal signs of cognitive alterations in FXPCs? b) What is the nature of language disfluencies patterns in FXPCs and are these related to a primary language deficit?). We will first give an overview of the theoretical background of the study in 2.2, focusing on defining the main processes investigated in Experiment 1. Subsequently, we will move on to describe the materials and methods in 2.3. Lastly, we will report the results obtained in 2.4 and we will discuss their interpretation in the last paragraph 2.5.

2.2 Theoretical background of the current study

As mentioned in 1.4.3, research focusing on identifying specific patterns of cognitive decline in language, particularly in neurodegeneration, seems to be promising in biomarker discovery as the study of language is non-invasive and can be potentially related to several disease specific mechanisms, as for example the potential basal ganglia involvement in the action naming deficit in PD. The most studied diseases are the most common ones, AD and PD primarily, and the techniques used to study them have been then extended to the study of other less common neurodegenerative conditions. This is what we aim to do with this study, as we believe it is fundamental to further explore differences among FXPCs, but most of all to investigate potential non-invasive measures that could advance the identification of prodromal signs of cognitive decline. We highlighted in Chapter 1 that research in this field has already identified potential identifiable patterns, such as language disfluencies, but the causes behind the presence of disfluent language are unclear [8]. Therefore, we will continue this line of study, by investigating the presence of potential disfluencies in our sample of FXPCs, but we will also pair this with other linguistic tasks that are more

direct measures of a language specific process that when disrupted, could contribute to produce disfluencies; this process is called lexical access.

2.2.1 Lexical access

We began the first chapter with the famous Shakespeare quote “*What’s in a word*” to emphasise the importance of the correct use of words, particularly of terminology in scientific research and of understanding the definitions and the implications. We would like to go back to this quote and link it as a *fil rouge* to the importance of words’ representation and meaning.

A word can be defined as:

“A speech sound or series of speech sounds that symbolizes and communicates a meaning”^{xvii}

The process we avail of to access the meaning, and the mental representation of a word is seemingly effortful, and relatively automatic, but this task is actually very complex as it imposes a high demand on our cognitive abilities and understanding how it works is fundamental to uncover mechanisms that could be involved in difficulties in language abilities. The words an individual knows constitute what is called the mental lexicon, which is a part of long-term memory. Although we need to store some sort of representation in the brain of the words’ form and meaning, there is no consensus in the literature on the way in which we access it. One of the main questions in the study of mental processes influencing language is how this mental lexicon is accessed by individuals. The process can be separated into two parts, that roughly refer to language comprehension (in spoken word recognition) and language production (spoken word production). We will briefly outline the main models as presented in the Oxford Handbook of Psycholinguistics [121] that in the history of psycholinguistics attempted to explain this process, both in comprehension and production.

Language comprehension: spoken word recognition.

Several models tried to answer the question “how does accessing the mental lexicon work?” The main models can be divided in three main groups: first-

^{xvii}<https://www.merriamwebster.com/dictionary/word#:~:text=%3A%20a%20written%20or%20printed%20character,characters%20representing%20a%20spoken%20word>

generation models, connectionist models and new approaches. One of the first models that attempted to explain this process was suggested by Forster. In this model words are organised in different bins with access files based on the way in which the word is presented, orthographic, phonological, semantic/syntactic. This model emphasises the importance of word frequency as it suggests that most frequent words are accessed faster as they were stored at the top of the bins. Forster suggests that this model can account for both written and spoken language, an aspect that many other subsequent theories challenge [203]. A second influencing theory was the *cohort model*. In this theory the most important characteristic is the idea that a word can be accessed directly without the intermediate steps of assembling all the phonemes, a step considered fundamental by many language theories before the cohort model. The work of Marslen-Wilson reversed this idea. They ran a series of experiments demonstrating that similar phoneme strings at the beginning of a word would activate several words with similar sounds (a cohort that did not need to have to assemble all the phonemes to activate the word). When additional information of the target word was available then all the competitors that do not correspond to the target word drop out, until there is enough information to correctly activate the word [204]. An aspect that was highly debated about this theory was the idea that the number of activated words in the cohort did not affect retrieval. In later versions this vision was changed, as basing the whole recognition of a word on a unique point, such as a specific phoneme string, does not seem to work with short words, whose unique distinctive point could be placed after the end of the short word (cat-cathedral); therefore the unique parts of a word cannot be the only feature used to distinguish between words [205]. Another important first-generation model is the *neighbourhood activation model* proposed by Luce and Pisoni [206]. This model's basic idea is very similar to the cohort theory, as it assumes that an acoustic phonetic input activates a group of word, called a neighbourhood, but this time the words are not connected because of the initial sound of the word, but competition for activation of each word is based on the frequency of the word, the number of words in the neighbourhood (neighbourhood density) and the frequency of the neighbourhood (neighbourhood frequency) [207]. Connectionist models, also called interactive activation approaches, are instead based on the idea of recreating an artificial neural network to mimic the human brain, and they are more focused on the

process rather than representation. In these models the units are connected through excitatory and inhibitory processes, progressively activating the units that best match the input, and the ones that do not match it [208].

One of the main models of this approach is the *TRACE model*, created by McClelland and Elman (1986) [209]. The *TRACE model* is based on the existence of many units organised in different interactive layers, the acoustic phonetic, the phonemes and the words. Each unit would be excited or inhibited based on how well the unit represents the speech input. Each unit in the model is replicated over each time “slice”, meaning that the input is effectively reiterated over and over to be spread over the layers until word activation, and this activation process is called “the trace” [209]. This model was criticised because of worries that it would not really reflect the mental representation of word recognition and that more efficient models are needed. Some of the criticism targeted the lack of inclusion of the word frequency effect (most frequent words are recognised faster than less frequent) and the interactive nature of the model, where potentially each layer could influence one another [210]. A second popular connectionist model was the *Shortlist model* proposed by Norris [211]. This model is very similar to the *TRACE model*, but the main difference is that the interactive activation is only “feed forward” and did not allow the reverse process [212]. The two models are very similar in terms of the behavioural mechanisms they could account for, and the debate between the most accurate hypothesis between feedback and feed-forward activation model was never fully resolved. Despite the criticism the *TRACE model* was highly influential and produced several experiments and publications, and despite the limitation that it actually did not really account for the mental organisation of word retrieval process, had the merit of focusing and showing the importance of using computational models to understand psycholinguistic processes [121]. The real nature of interaction between units both at a prelexical and at a lexical level is still unclear, therefore the field has produced new theories over the last few years, and despite being promising they still need to be adequately investigated to be refined and able to account for all the many different processes. A very interesting model is the *Bayesian model*. This approach is mainly visible in the model called Shortlist B, which is very similar to the first model, but uses a Bayesian framework; instead of interactive activation over time used by the connectionists model mentioned above, this approach is based on the idea that

individuals use decision making processes based on probability to recognise the most probable and optimal decision, in this case the most probable word to recognise [213].

Another interesting model is the one based on *network science hypothesis*, a branch that encompasses different fields which hypothesises the existence of a network structure that is made up of nodes, connected between each other through links [214]. Applied to the word recognition process, it means that the words are the nodes and links are made if the words are related (phonologically or semantically). The main idea in network science is based on the ecosystems of the network, hence on the delicate architecture of it rather than the process, and it assumes that a specific process can work in a single network but not necessarily in another network as they may have different structures and not function in the same way [215]. Despite going against most of the findings in spoken word recognition, as research on language is usually based on understanding a process that could be universal, this model has the merit of explaining the importance of the structure, and of hypothesising differences in different language systems, as shown in the literature [216]. One of the most innovative approaches is the one proposed by Baayen, Shaoul, Wilitis and Ramscar in 2006 (*WALK model*) [217]. They take as the main unit a triphone that becomes a lexeme in the output, and it is constantly updated with experience in different contexts working in their theory as an error trial algorithm. The major difference is that they do not use the word as the basic unit; in fact, they do not conceptualise the existence of “words” representation, rejecting the idea of different levels between word segmentation and word learning. In this model learning is inherently part of the model, allowing the model to adapt to different kinds of situation including mispronunciations and different accents[217].

Language production

Successful communication can be defined as effectively producing a message that someone wants to convey, and this is achieved through language production. Ever since babies begin to produce the first words between the first and second year of age, this ability grows and changes during their life. We have seen in the previous paragraph how a message is received and successfully understood, we will now deal with the opposite process, how words are retrieved to become the output. Many theories and models deal with language production, both in

psycholinguistics, neurolinguistics, and motor speech theories. We will now summarise the main models dealing with spoken word production as outlined in the above-mentioned Handbook [121].

Spoken word production refers to the ability of retrieving the correct word from the brain and enhancing a series of mechanisms that will lead to the production of that word [218]. All models agree on the existence of different stages encompassing semantic and lexical levels, followed by morphological and phonological aspects [121]. Semantic refers to the meaning of the word, i.e. the message that the speaker wants to convey. One of the main questions regarding the process of retrieval of the word regards the representation that we have in the brain of this word, the lexical representation that involves both the semantic aspects (the meaning) and the syntactic aspects ^{xviii}. How are words represented in the mental lexicon? Models of words' representation in the brain can be divided in decompositional or non decompositional. The first type of models assumes that a word meaning is decomposed in different features that can be shared among different words and concepts. In this interpretation what makes a concept individual, is the unique combination of different features. A foundational model in this area is the one proposed by Taft [219]. On the contrary, in non decompositional models, words have an almost perfect correspondence with the lexical concepts, so the lexical representation is identical to the word, like in the model proposed by Roelofs [220]. Both models agree on believing that the organisation of the mental lexicon corresponds to the organisation of concepts in semantic memory; in this kind of memory, concepts are organised in a network, but they differ in the way the connections are made between words [121]. In the non decompositional model the connection depends on the strength of the connection between concepts; in the decompositional models it depends on features that overlaps between concepts. The main question is whether there is a one-to-one relationship between one's mental lexicon and the external world. The debate is still open and cross linguistic studies are challenging the view of a perfect correspondence between prelinguistic and

^{xviii} Syntax refers to those representations that convey certain aspects of words at the sentence level regarding for examples the order of the words in a sentence, or the grammatical categories, aspects that are discussed in models of language production because it is important to understand how syntactic aspects are stored in the mental lexicon and therefore retrieved, as single words are not spoken in isolation.

linguistic representations [221]. Despite the differences, both models agree on the activation of the words that in turn activates semantic neighbours. The activation is discussed in models of lexical access which also involve discussion about the syntactic properties of words. For example the distinction between grammatical categories, such as gender and number, are discussed by these theories [222]. The first and most important model for lexical access in language production is the one proposed by Levelt [223]. This model suggests the existence of 2 stages: lexical concepts which are connected to lemmas, a node that includes language specific syntactic information; and then lemmas are linked to lexemes, nodes that includes language specific phonological aspect of the words, the word form. The first stage is the one that deals with syntactic information such as grammatical categories, gender and number; the second stage deals with the word form. Lemmas are believed to be halfway between the concept and the language representation. The idea of lemmas and lexemes is still debated as not everyone believes they exist. The lemma/lexeme distinction is challenged by those who believed that it is not necessary to access syntactic information to reach phonological information first; for example, Caramazza is one of the main exponents of this idea [224]. In general models of word production, speakers usually see an object and name it as fast as possible (average is 600ms when unprimed and 100ms faster when primed) [121]. There are certain variables influencing accuracy and speed, particularly frequency [225] and age of acquisition of the word [226] are considered the most important. This is based on the idea that when a specific pathway to retrieve a word is used more often, the word is activated faster than the others. It is now well established that words retrieved from a rich mental lexicon are interconnected, and that the process of retrieving a word necessarily entails activations of other words, whether they are semantically or phonologically connected [227]. The question arising here is if the process is competitive or non-competitive. There is evidence to support both accounts. The question has to do with how we can retrieve the appropriate word in the huge network of the mental lexicon. Some theories suggest that non target words can delay the retrieving times; that for example a semantic distractor could interfere with retrieving the target word, hence delaying the whole process[228]. The hypothesis is that competitors delay retrieving time because they compete for selection and semantic similarity is supposed to increase latencies. This competition is assumed to happen at the

lexical level when the item is selected. But semantic distractors do not always generate delays or real competition, as for example in some experiments semantic or thematic associates appear to facilitate retrieval instead of delaying it [229]. The hypothesis of why delays occur then is that in the experimental setting, a group of target words do cause delays but at a post lexical level, when the examinee needs to remove the non-target word. The other important matter regards whether the process is serial or interactive. In serial models, the information is supposed to flow from concepts to lemmas then lexemes and then phonology, and that each process must be completed before passing to the next one and it is unidirectional. Other serial models allow activation of the next level even before the process is completed at the higher level. In interactive models the information can flow in both directions with feedback also coming from lower levels [230]. Middle ground theories suggest that the system is modular and there is a certain extent of interaction between levels, but in a small amount because the overall goal is the selection of a target word [121].

The dissociation nouns-verbs

The different models on lexical access and how the word is represented in the brain, and whether there is a one-to-one correspondence with the real word and a different storage systems per word, or grammatical class or not, is particularly interesting for the purposes of this study. As we have mentioned in the literature review of language and neurodegeneration, there seem to be differences in the preservation of different word classes in the different neurodegenerative disorders, particularly in verb (action words) and noun (objects words) dissociations. A considerable amount of evidence supports the idea of a difference in brain organisation for verbs and nouns, and the picture naming task is a particularly useful tool to investigate this difference. For example, Broca's aphasics often result in having higher difficulties in production of verbs compared to objects, while Wernicke's aphasics display more difficulties with the opposite pattern, naming objects compared to verbs, and interestingly this dissociation has been demonstrated to be present across languages [231]. The interpretation of this dissociation is still controversial though, as some researchers support a strict class distinction in the brain, with nouns being represented in one region and verbs being represented in another; Caramazza and Shapiro are among the main exponents of this theory. According to this view

left temporal areas would be more involved in noun representation, while left frontal areas would be engaged by verb representation [232]. Other theories suggest that this dissociation does not exist per se in the brain; instead what counts is the meaning conveyed by the verb or the noun and the way they are processed, and therefore more related to semantic properties as functionalist approaches suggest [233]. According to this second group of theories, the dissociation could be more related to differences linked for example to sensorimotor representations, as action names engage more areas connected to the motor cortex, and object names involve more areas connected to the visual cortex. Additionally, they point out that the difference might also be related to the inherent properties of actions and objects such as frequency variations, age of acquisition, the number of attributes and neighbourhood evoked by objects and actions, and the potential lexical competitors [231]. Lastly, evidence for some sort of dissociation comes also from studies investigating language acquisition in children, as there seems to be consensus on the fact that across languages, objects names are acquired first, probably because verbs and the ability to predicate are necessarily and inherently more difficult, and children need to master the attribute of objects and the related semantic system before being able to extract rules to be attributed to verbs, and also adjectives for example, as both classes are necessarily dependent on nouns [115]. Despite the consensus of some sort of dissociation, whether linked to the word class or to the words' semantic aspects, the picture depicted by literature in the field is quite complex, because each view has also different groups of theories. For example, among those who believe in a segregation due to the grammatical class there is a debate on when the segregation occurs, at what level of word retrieval: the more lexicalist view believes that this separation happens quite early, and that grammatical and syntactic aspects are retrieved at the same time as the lexical aspects. In this view the segregation should occur both when words are produced or in comprehension settings, and both when they are in context or isolated; this is compatible with the view of a segregation in neural networks of the word class [234]. The second set of theories believes that grammatical class is not connected to lexical aspects, and it is acquired and retrieved through a system that is not connected to lexical retrieval but separated and applied during processes of sentences. This is usually based on Generativist approaches which try to find universal applicable definitions across languages, and they do believe that

meaning alone cannot distinguish between lexical categories, and that the crucial distinction happens at a sentence level. In their view, grammar and syntax take meaning when put together with other sentences elements, and this view is compatible with the idea that there is not a segregation in neural networks per word class, but rather for processing and integrating nouns into sentences and verbs into sentences, therefore the dissociation should only be visible at a sentence level [235]. Lastly the Emergentist view believes that grammatical knowledge is not important in either level, lexical or sentence level. They suggest instead that some constraints, such as prototypical nouns referring to objects and prototypical verbs referring to events, or the presence of phonological similarities amongst nouns and verbs morphology, contribute to the dissociation. Furthermore, they do not believe in a complete segregation of neural networks, in this theory the same network would be engaged for both, and the extent would depend on the properties, the constraints evoked and the task demand. However they accept the hypothesis of partial separation of temporal and frontal network[233, 235]. Despite the controversy, the presence of a dissociation is evident and whether this dissociation occurs because of the inherent properties of the word or sentence, similar areas are identified to be engaged during action and object representation [182]. That is probably why this distinction has been found in neurodegenerative disorders and why it can be important to further investigate, as it can be useful to point to specific areas that might be affected, even in behavioural studies that do not directly involve neuroimaging or electrophysiology. Language seems to resist well to decline in aging; however, some aspects start to be impaired both in language production and comprehension as we get older. Particularly production is an aspect that gets compromised especially after the age of 80 [236]. Lexical access seems to be affected in older individuals, and if we consider the different levels and stages involved in lexical access this is quite understandable as complex tasks become more and more difficult as we age, for example the activation of the network, selection and inhibition of competitors might be a particularly complex task. Lexical access difficulties have been registered since the age of 50 and become more common after the age of 70 [237]. Particularly, accessing semantic representations seems to be relatively unimpacted by age, while the stage that requires accessing the phonological representation often causes delays in RTs and less accuracy, leading to the so called tip-of-the-tongue (TOT) experience,

where people seem to have a sensation of what they want to say but can't say it because the phonological form of the word is unavailable. Yet they still can access the semantic representation, which is typically stored in long term memory, as opposed to the more formal aspects, usually stored in the phonological working memory [238]. Despite the normal difficulties that anyone can encounter with healthy aging, we're interested in this study in understanding any potential difference between healthy aging and a group at risk of developing cognitive decline, hence whether individual differences can affect the process: as we have mentioned previously several aspects of language can be impaired in neurodegeneration, and we would like to understand whether this is detectable at a prodromal stage and whether this is linked to lexical access and specifically to the dissociation between nouns and verbs.

In the next sections we will discuss the first two research questions, addressed by the linguistic experiment 1:

- a) Is it possible to identify potential differences between FXPCs and a control group through language assessment that could indicate prodromal signs of cognitive alterations in FXPCs?
- b) What is the nature of language disfluencies patterns in FXPCs and are these related to a primary language deficit?

We hypothesise that some differences will be identified between the two groups; particularly that the FXPCs group will show a more disfluent language pattern, as previously seen in literature, compared to the control group, and that this might potentially correlate to the performance in the tasks tapping directly into the lexical access process, indicating disruption to that process as a potential cause for disfluencies in language.

2.3 Materials and methods:

2.3.1 Participants

A total of 121 participants were enrolled in the study, 70 Fragile X premutation carriers (FXPCs) and 51 control participants (CP). FXPCs were included based on a confirmed diagnosis of a premutation in the *FMR1* gene and being over 18 years of age. Control participants were matched by age to the FXPCs group, with no family history of *FMR1* gene mutations and no indication of carrying the premutation. Both the FXPCs and control groups were recruited through patient

associations, as outlined in 1.6, and through advertisements at higher education institutions. Particularly, the International Fragile X Premutation Registry proved particularly helpful in recruitment efforts [33]. Ethical approval was granted by the School of Psychology Ethical Committee at Trinity College Dublin, and all participants provided written informed consent. Originally designed to be conducted in-person only, the study later was expanded to include online assessment too due to limited number of participants recruited.

As shown in Table 8 the total sample consisted of 121 participants, 99 females and 22 males, who were divided into two groups: FXPCs and CP. The mean age of the participants across the entire sample was 45.57 years (SD = 14.68, SEM = 1.33). In the in-person group (n = 31), 14 FXPCs (all female) had a mean age of 48.57 years, while 17 CP (13 females, 4 males) had a mean age of 33.11 years. In the online group (n = 90), 56 FXPCs (47 females, 9 males) had a mean age of 49 years, and 34 CP (25 females, 9 males) had a mean age of 49.85 years. Combining both in-person and online participants, the 70 FXPCs had a mean age of 48.91 years, while the 51 CP had a mean age of 44.27 years. Most participants reported taking medications and had a history of neurological, neurodevelopmental, or mental health disorders.

	FXPCs	CP	Total
In-person	14 Females/ 0 Males	13 Females/ 4 males	27 Females / 4 Males
Online	47 Females/ 9 Males	25 Females / 9 Males	72 Females/ 18 Males
Total	61 Females/ 9 Males	38 Females/ 13 Males	99 Females / 22 Males

Table 8: Demographic characteristics of the population enrolled in the study.

Overview of the population's characteristics, expressed as participants recruited for the in- study in Ireland, participants for the online study, and total number of participants

The control group and FXPCs group primarily consisted of native English speakers, with the exception of two participants in the FXPCs group (one recruited online and one in-person) that were age matched with two non-native speakers' participants in the control group (one online and one in-person). Both groups were similar in terms of age, education level (with all participants having at least third-level education, except for two in the FXPCs group and three in the

control group), and gender distribution. Given the higher prevalence of the premutation in females, the majority of participants were female, though males FXPCs were also recruited to explore potential gender differences. Among the males FXPCs, two participants were diagnosed with FXTAS during the study. Additionally, the group exhibited a history of self-reported or diagnosed neurological conditions, aligning with features of FXPAC and FXAND (outlined in Table 1). All participants provided a comprehensive set of demographic and clinical information, which served as independent variables for the analysis, potentially influencing performance on cognitively demanding tasks. The collected data included: age; sex assigned at birth; the number of CGG repeat expansions specific to the FXPCs group; linguistic background (including the number of languages spoken, fluency levels, and bilingual status); educational background and field of study; use of medications (specifically psychoactive agents such as antidepressants, antipsychotics, benzodiazepines, and treatments for attention deficit hyperactivity disorder, including methylphenidate, amphetamines, tomoxetine, and guanfacine, as well as steroid derivatives like pregnenolone, progesterone, estradiol, DHEA, allopregnanolone, and ganaxolone, along with a complete list of any other medications); and any previous medical history of neurodevelopmental disorders, neurological disorders, migraines, mental health conditions (including depression, schizophrenia, autism spectrum disorder, or attention deficit hyperactivity disorder), or traumatic brain injury. Additionally, information regarding current pregnancy status or the use of hormonal contraceptive methods was collected.

2.3.2 Procedure

In-person study:

All research participants involved in the in-person part of the study were assessed at the Trinity College Institute of Neuroscience (TCIN) in Trinity College Dublin. Blood samples were collected from each participant, with the procedure conducted by the PhD candidate^{xix}, another trained phlebotomist, or a research nurse from the Saint James Hospital Research Facilities. A single 9 mL vial was obtained per participant and processed to extract plasma within one hour of collection by a trained research assistant at Ulysses Neuroscience Ltd.

^{xix} The PhD candidate underwent medical Phlebotomy training to be able to perform the procedure and accelerate the recruitment process.

Subsequently, the blood samples were transported to the laboratory facilities at the headquarters of Ulysses Neuroscience Ltd. The entire blood sampling process took approximately 15 minutes. The methodologies and results concerning to the analysis of plasma-based proteins will be detailed in 3.2.2. Following blood sample collection, each participant was monitored for a period of 15 minutes before being administered psycholinguistic tasks as part of the language assessment by the PhD researcher. Participants completed three linguistic tasks individually in a quiet, well-lit room, using a standard computer screen positioned approximately 80 cm away. This setup followed the procedural guidelines determined by the normative studies of the International Picture Naming Database (IPNP)[231]. The language assessment lasted between 25 and 30 minutes per participant. Participants were allowed to have breaks between each task, however none of the participants availed of this option.

Online study:

All research participants involved in the online study were provided with a link to join a Zoom call with the PhD researcher, who attended each session from start to finish to address any questions or concerns. During the call, participants received links to access the online tasks and followed the instructions provided by the PhD researcher to initiate the online experiment. Identical instructions for completing the three language tasks were given to both in-person and online participants, ensuring a comparable total duration of linguistic performance for both groups. Detailed descriptions of each task will be presented in the materials section below.

2.3.3 Materials

The picture naming task

This task was selected to evaluate language production, as it is widely used in literature to investigate potential delays and errors in lexical retrieval, a complex process discussed in 2.2.1, which may be compromised by various factors, particularly changes in brain networks associated with neurodegeneration. The task serves as a particularly appropriate paradigm for studying real-time language processing and is recognised as the first psycholinguistic task employed to examine these types of processes [239]. We designed a timed

picture naming task to assess (a) latency and (b) accuracy^{xx} in FXPCs compared to a control group. Drawing upon the potential differences in action and object organisation in the brain, as discussed in 2.2.1, and existing studies that have identified distinct patterns in naming actions and objects within neurodegenerative disorders, we employed the same paradigm for the task design. The stimuli were divided into two matched groups: one for object retrieval and the other for action retrieval. The design was done following the norms and paradigms established by the International Picture Naming Database (IPNP), developed by the Centre for Research in Language at the University of California, San Diego. The primary objective of this project was to create a database for future cross-linguistic studies, with stimuli validated across various variables known to influence picture naming tasks [135]. The database is made-up of 520 black and white drawings of objects and 275 actions. Norms for a number of psycholinguistics variables are indicated for each picture, and they are consultable online through a database query. As discussed in the literature review on language and neurodegeneration, a significant limitation in studies examining differences in object and action naming within these disorders is the lack of matching for psycholinguistic variables that are known to influence performance on this task. Consequently, during the design phase of our study, we ensured that each individual picture was matched one-to-one across a series of important variables:

- **Age of Acquisition (ecdi):** These variable measures the age at which the selected words are typically acquired (before or after 3 years of age).
- **Log Word Frequency(efreq):** This represents the frequency of each word within the specified language.
- **Semantic Set Size:** This classification applies to the semantic group to which a word belongs (e.g., people, animals, foods, etc.).
- **Word Length:** This refers to the length of the word, measured in syllables.

^{xx} Latency= reaction times
Accuracy= % of errors

- **Presence or Absence of a Fricative:** This variable indicates whether a fricative consonant (such as /f/, /v/, etc.) is present at the beginning of the word.
- **Name Agreement(elex1):** This assesses the degree of correspondence between the picture and the name assigned to it by participants in the normative studies.
- **Object complexity (Ovcjpg):** this measure estimates the visual complexity of the image. The complexity score has been defined and validated by the IPNP database.^{xxi}

A complete list of words and their associated variables can be found in Appendix 1: Appendix 2: . The matching procedure of psycholinguistic variables between conditions was conducted manually by the researcher, resulting in a task composed of 60 objects and 60 actions.^{xxii} Despite considerable effort, achieving perfect matches for all the variables of interest across all pictures proved impossible. Consequently, when certain variables were not matched, we prioritised name agreement, frequency, and age of acquisition, as these are regarded as the primary features influencing performance on the task in studies using the database [231]. As the authors of the database suggest, it may not be feasible to achieve perfect matches between actions and objects across all relevant parameters, given their inherently and naturally different characteristics [231]. The task was developed using the Python-based software PsychoPy.

^{xxi} Estimates of object visual complexity was obtained by the picture itself converting the picture into various graphical files with an Image software and then complexity was analysed as defined in the IPNP database. <https://crl.ucsd.edu/experiments/ipnp/dataquery/queryfull.php>

^{xxii} The object conditions consisted of 61 trials because the word “tree” was accidentally repeated. The second occurrence of the word was excluded from the analysis for all participants.

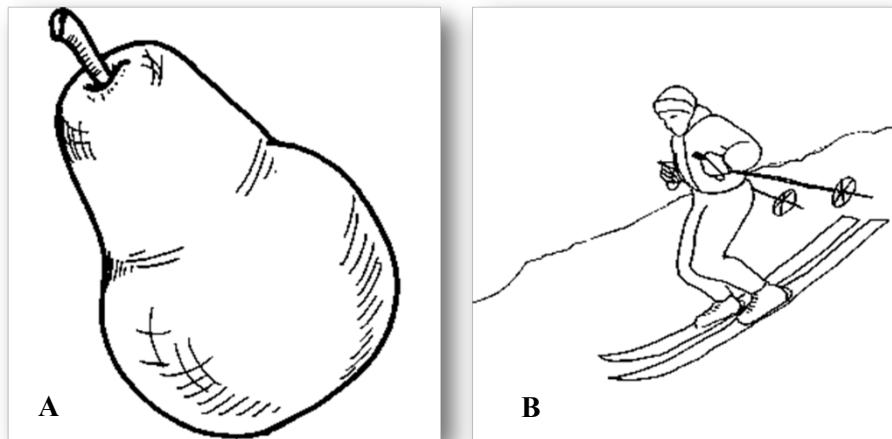


Figure 3: representative stimuli for the picture naming task.

(A): object condition task. Participants are presented with a sequence of picture representing objects and they are asked to name it as fast as they can. The target word for this picture is “Pear”. (B): Participants are presented with a sequence of picture representing actions and they are asked to name it as fast as they can. The target word for the example action in the picture is “Ski/Skiing”.

The stimuli were categorised into two distinct tasks, one for objects and another for actions (Figure 3), designed in accordance with the settings used in the original normative studies performed to establish the database [135]. Extensive troubleshooting was required over several months to successfully implement the tasks, as the voice response function was not initially integrated correctly within the software. Initially, the microphone was not recognised by the software, requiring the testing of various microphones with different specifications. Subsequently, several modifications to the script were necessary to ensure that separate recordings were obtained for each trial, a crucial step for the accurate analysis of the audio files. In contrast to other similar psychology software, PsychoPy does not offer the capability to automatically generate voice onset RTs. Therefore, both manual analysis of each audio recording and automated analysis via a specifically designed algorithm were employed to detect onset times. These aspects will be further elaborated in the analysis and results section 2.4.

In-person procedure

The procedure followed the guidelines established in the International Picture Naming Database (IPNP) [231]. Participants were seated in a chair approximately 80 cm from the screen. Prior to commencing the experiment, the researcher provided an overview of all tasks and instructions on how to perform them, highlighting that written instructions would be available at the beginning

of each task. Each task was preceded by a practice session containing five pictures for each condition (five objects and five actions). Participants were provided with the following instructions for both sessions, indicating whether they were about to start the "objects" or "actions" condition, to minimise confusion about the word class (object or action):

“Welcome to the picture naming task! In this task a sequence of drawings depicting objects/actions^{xxiii} will appear on your screen. First, you will see a fixation cross, you do not need to name it. Then, the pictures will appear in sequence. Name each picture as quickly as you can avoiding coughs, false starts, articles and any extraneous material or anything other than the best and shortest name you could think of for the depicted object/action. Press the spacebar when you’re ready.”

A within-subjects design was employed for this study, where all participants performed both the object and action sessions. The images were centred on the computer screen, and prior to each task, a fixation cross was displayed for 200 milliseconds, followed by a 500-millisecond interval. Each picture remained on the screen for 3000 milliseconds, but participants were allowed up to 4000 milliseconds to name the target image. The interval between each picture presentation was randomised to prevent habituation, ranging from 1000 to 2000 milliseconds [135, 231, 240]. The experiment was designed to enable the software to record each trial of the target picture individually and to generate separate WAV files for each image named. The onset of the picture display was synchronised with the beginning of the trial and the audio recording to facilitate the analysis of RTs. One picture was accidentally repeated among the trials; however, the second occurrence of it was subsequently excluded from the analysis, and no picture was pre-exposed, thus eliminating any potential training effects associated with the stimuli. The experimental setup included an external microphone to enhance the audio recording quality, which was regulated to maintain a consistent distance from each participant's mouth. During each session, the experimenter documented the correct and incorrect responses, which were later analysed by an independent scorer through review of the recordings. Each part of the tasks lasted less than five minutes. Prior to the beginning of each

^{xxiii} Depending on the session they were about to perform the word “object” or “action” was displayed in the instructions.

subsequent session, participants were provided with instructions once again and given the option to take a break if needed. For each participant, the object session was conducted before the action session.

The Visual Word Paradigm

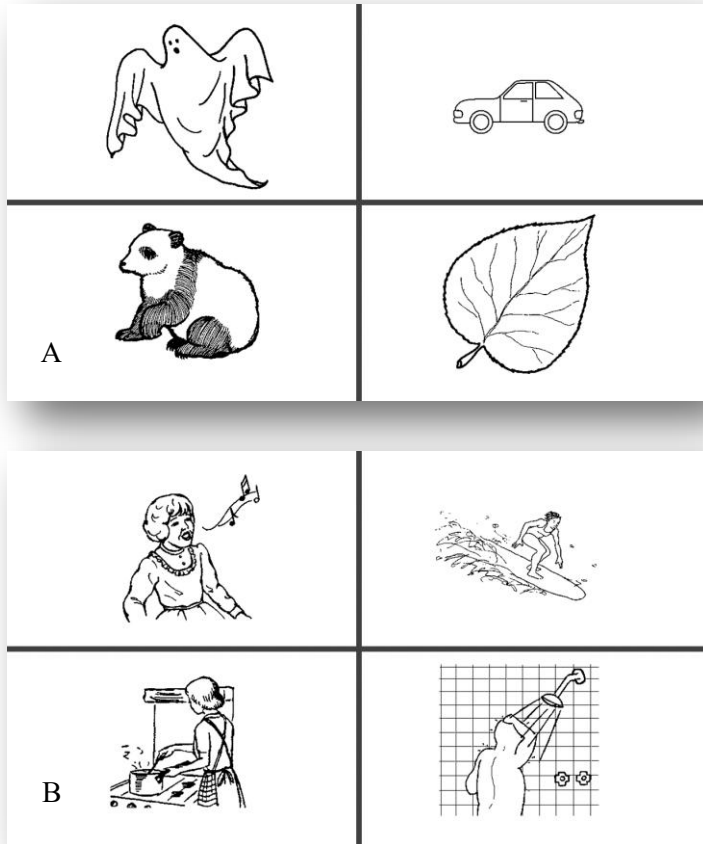


Figure 4: Representative stimuli of the Visual Word Paradigm.

A: object condition task: when the auditory prompt names “Panda” participants have to click on the correct object picture. B action condition task: when the auditory prompt names “Surf” the participant has to click on the correct action picture.

This task consisted in a set of 4 pictures (Figure 4) presented on the screen accompanied by an auditory prompt in which a voice named one of the pictures on the screen. Participants were required to click on the corresponding target response using a mouse. The audio files were recorded by a native speaker who is an experienced coach in storytelling and vocal exercises. To ensure consistency, all audio recordings of the target words were edited to initiate the voice onset at 3000 milliseconds, thus standardising the conditions for each auditory prompt. Additionally, the recording of participants' responses by the software started immediately after the auditory prompt, further contributing to the consistency of the experimental conditions. The same database and

psycholinguistic variables employed in the picture naming task were also utilised for this task. The object part included 22 trials, while the action part included 23 trials and they were both preceded by a practice session for each condition, consisting of 5 trials each. The target words in both the object and action conditions were matched according to age of acquisition, as categorised by the IPNP database, with all words classified as level 1 (acquired within the first year of life). Additionally, the words were matched for frequency, ranging from 2 to 4 according to the CELEX database of the IPNP. In the object condition, half of the target words were animated objects, while the other half were inanimate objects, because the two categories are believed to be processed differently, especially if presented visually [241]. The 4 words presented in each trial were controlled for variables known to influence RTs in visual word recognition. They were matched ensuring to have different categories per each trial (people, animals, body parts, vehicles, foods, things to wear, small and large artifacts, objects and phenomena in nature[135]), similar frequency and that each word began with a distinct phoneme to mitigate any potential facilitations or delays in word retrieval [242]. Because the database had a limited number of action picture items, it was not always possible to match actions across the same variables used for objects. As a result, priority was given to matching based on frequency, age of acquisition, and differences in onset phonemes [231]. In-person participants completed the Visual Word Paradigm task immediately following the picture naming task, with the object condition presented first, followed by the action condition. The instructions displayed on the screen were as follows:

“Welcome to the Visual Word Paradigm!

In this task you will see 4 pictures on the screen depicting actions^{xxiv}. You will hear a word describing one of the 4 images. Use the mouse to click on the image depicting what you heard as quick as you can. Press the spacebar when you’re ready to start! Press the spacebar when you are ready to start!”

Following the instructions, participants started performing the task using the equipment provided in the room, including a mouse, keyboard, and computer screen. The duration of each part of the task was approximately two minutes.

^{xxiv} Depending on the session the word “object” or “action” was displayed in the instructions.

Online experiment

Picture naming and Visual Word Paradigm

The online versions of both the Picture Naming task and the Visual Word Paradigm were conducted in a way closely resembling the in-person sessions, and participants were given identical instructions in both conditions. The various tasks were uploaded to the Pavlovia platform using the PsychoPy builder view. This procedure automatically translates the Python-based script developed in PsychoPy into JavaScript, which is necessary for uploading the experiments online. After troubleshooting several errors encountered during the translation from Python to JavaScript, we successfully uploaded the experiments. In the Picture Naming task, one picture from the practice session of the object condition had to be replaced, as the platform was unable to recognise the features of the original image. However, since the testing pictures were not scored or assessed and were not matched for any variables, we did not anticipate any impact on performance in the main tasks, which remained identical to the in-person version. To closely replicate an in-person session, the language assessment was conducted via a Zoom call with the PhD researcher. Participants received detailed written instructions via email to prepare their computer devices prior to the call, ensuring they had a keyboard, a mouse, and that their device was equipped with a built-in microphone. Additional recommendations were provided regarding browsers that would function better with the Pavlovia platform.

Participants were instructed not to use any device other than a computer or laptop for the assessment. Before the call, participants received all relevant links to perform the assessment and were advised not to open them prior to the call. Considering that many FXPCs experience elevated levels of anxiety, which may manifest as difficulties with technological tools, they were permitted to access the picture naming practice object link to familiarise themselves with the five pictures before joining the call. Most participants navigated the links without issue; however, some required guidance from the researcher regarding microphone setup or audio settings on their devices. Ultimately, all participants successfully completed each task. Following the completion of each task, participants were prompted to open the subsequent link or take a break if needed.

The overall duration of the assessment was comparable to that of the in-person sessions.

The picture description

At the end of the language assessment, participants were administered a picture description task. The "Cookies Theft" picture from the Boston Diagnostic Aphasia Examination-(BDAE-3)[243] was chosen, as it is regarded as one of the most widely used tools in clinical language analysis. The picture was displayed on the screen, and participants were instructed to describe “everything that you see going on in the picture,” in accordance with the instructions provided in the Boston Naming Test [243].



Figure 5: The Cookie Theft picture from the Boston Diagnostic Aphasia Examination.

The picture is taken from the Boston Diagnostic Aphasia Examination-Third Edition reprinted with permission from PRO-ED, Inc. [243]

The PhD researcher did not provide any further details on what to describe the image; instead, she prompted participants by asking, “Is there anything else you would like to add?” if participants displayed difficulties in understanding the task or if their descriptions focused exclusively on a small portion of the picture. Additional prompts were not given when participants exhibited a high level of anxiety during the task. The responses of in-person participants were recorded locally on the computer running the experiment. Each audio file was then labelled with the participant’s code and prepared for analysis (converted to WAV

format and adjusted to meet the requirements of the analysis software). For online participants, descriptions were recorded via Zoom and subsequently prepared for analysis.

2.3.4 Analysis

The analysis of the collected samples aimed to address the core research question of the study: can differences between FXPCs and CP be detected through language assessments? We hypothesised that differences between groups could be found in the language production task, the picture naming task (in latency and accuracy), in the language reception task (the visual word paradigm), and in the picture description task, particularly in the disfluency measures. In the following sections we will first outline the process performed for data review and processing, and then we will outline the statistical methods used. Statistical analyses were conducted using InVivoStat (v4.3.0) and GraphPad Prism (v10.1.2).

Data processing and review

Picture naming

The analysis of the audio files for each trial involved several steps to generate data suitable for statistical analysis. We conducted both quantitative and qualitative analyse, specifically, to assess latency (Reaction times, RTs) and identify potential errors or disfluencies (accuracy). The task was structured so that PsychoPy created a separate audio recording for each trial in a designated folder. However, as PsychoPy does not automatically record RTs, each audio file required individual analysis. With a total of 121 participants and 120 trials per individual, the dataset consisted of 14,520 audio recordings.

In-Person Data Collection:

When tasks were conducted locally using PsychoPy, the audio files were directly saved in a folder in wav. format, with each file named according to the participant's numerical ID to prevent confusion.

Online Data Collection:

For tasks conducted online via the Pavlovia platform, the audio recordings were temporarily stored on the platform, labelled with the participant's numerical ID and the trial name. The files were downloaded and organised into folders corresponding to each participant. Since Pavlovia saves audio files in webm

format, which is not compatible with the other software used for subsequent analysis, a Python script was created to automatically convert the files from webm to wav format. Once all files were converted to wav. format, both in-person and online recordings were processed in the same way.

Automated RT Analysis:

The voice onset RTs were initially measured using a MATLAB script, which used the built-in function *detectspeech* with a precomputed speech detection threshold.

Manual RT Verification:

Due to the variability in audio quality, particularly in online recordings where background noise was more prevalent, all audio files also underwent manual analysis. This was done using Audacity, a professional sound analysis software that enables background noise reduction and the visualisation of spectrograms or waveforms to accurately identify voice onset, as illustrated in Figure 6. The manually identified onset times were recorded in an Excel file for further analysis.

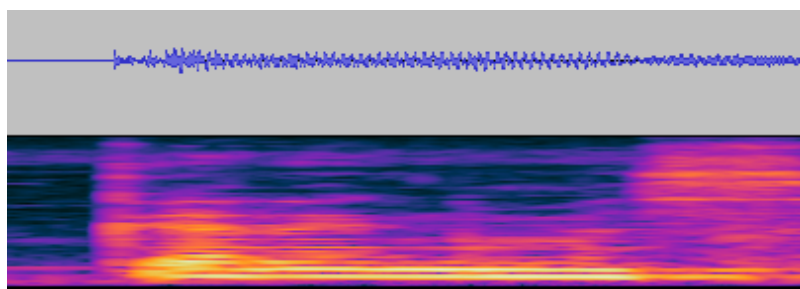


Figure 6: Waveform view and Spectrogram view of an occlusive consonant voice onset.

In this figure we show a waveform view (above) and a spectrogram view (below) of an occlusive consonant. A waveform is a visual representation of an audio signal showing how the amplitude changes over time. It shows “time” in the x-axis and amplitude (loudness) in the y-axis. A spectrogram is a visual representation of an audio signal and how it changes over time, used to see patterns in speech sounds here. The x-axis represents “time”, and the y-axis represents “frequency”. Occlusive consonants (p,t,k,b,d,g,) are sounds made by the complete obstruction of the vocal tract and interruption of the airflow.

After analysing the audio recordings from 43 participants (29 FXPCs and 14 controls), the same files were used to train an undergraduate student, a native English speaker with psycholinguistics training, in performing manual voice onset analysis. This training was conducted by the PhD candidate, who then assigned the student to function as an independent scorer. To ensure unbiased

analysis, the student conducted a blind review of the audio files, unaware of whether they belonged to FXPCs or control participants.

The student was also instructed to flag any potential errors or disfluencies in the Excel files containing the results. These flagged errors were subsequently rescored by the PhD candidate and compared with notes taken during data collection. Any uncertain cases were reviewed and resolved by the PhD candidate, who performed a final round of analysis reassessing all participants' RTs and scores. We chose to compute RTs for all responses, including those containing errors, to explore whether words with higher error rates were also associated with slower latencies. Voice onset RTs were defined as the onset of the first phoneme of a codable word, whether or not it was the correct target. In cases where the target response included a disfluency that did not qualify as the start of a word (e.g., filled pauses indicating planning difficulties), voice onset RTs were recorded from the onset of the first phoneme of the word. This approach was taken because filled pauses are generally considered a sign of planning difficulties [244], and we aimed to capture the exact time required to produce a word despite potential planning difficulties. The types of disfluencies and errors considered will be discussed in detail in the qualitative analysis section 2.4.3. To the best of our knowledge, this study is among the first to analyse disfluencies and errors in a picture-naming task involving FXPCs. Typically, responses containing errors, hesitations, pauses, or repetitions are excluded from picture-naming studies. However, since the literature indicates that FXPCs tend to produce more disfluencies than control participants in discourse settings [7, 8, 108], we wanted to determine whether they also exhibit disfluencies or extraneous material in single-word production contexts. The files were then analysed again to categorise the different type of errors and disfluencies as outlined in the qualitative analysis section. Each error type was first colour-coded and then assigned a numerical value, which was used to compute both the total number of errors of that type per participant and the total number of errors of that type per word.

Analysis of accuracy and qualitative analysis of type of errors

To investigate whether differences between groups were detectable in accuracy as well as latency, we performed qualitative analysis to measure the percentage of errors made by each group.

First, we aimed to examine potential overall differences in accuracy between the groups and across modalities. To achieve this, we statistically compared the total percentage of errors and disfluencies. Next, we conducted a qualitative analysis of the different types of errors, which will be outlined later in this section. Here, our primary focus was to identify any distinct patterns in the types of errors made by the groups and across modalities, rather than detecting statistical differences in error types between the groups. We identified four macro categories and eighteen subcategories of errors, using methodologies employed in similar studies within the literature, as outlined in Table 9 [115]. Table 9 categorises the errors into four primary categories, showing examples of errors made by participants: Semantic^{xxv}, Visual, Other, and Dysfluencies, each containing various subcategories that capture the nature of the errors.

Semantic errors are further subdivided into four types.

- The **superordinate** error occurs when a participant uses a broader term in place of a more specific one, such as substituting *bug* for *ant*.
- The **coordinate** error involves the use of a term that shares the same degree of specificity, e.g. replacing *lettuce* with *celery*.
- In contrast, the **subordinate** error requires using a more specific term, such as saying *bungalow* instead of *house*.
- Finally, the **thematic** error is characterised by the use of a term that is thematically related, as seen when a participant substitutes *screwdriver* for *drill*.

Visual errors include two distinct types.

- The **misperception** error appears when a participant misinterprets an image due to visual factors rather than semantic ones, such as identifying a *knot* as a *pretzel*.
- The **misinterpretation** error occurs when the participant focuses on a specific portion of the image, leading them to use a term semantically related to the target word but incorrect, like referring to a *chin* instead of a *woman*.

The category “**Other**” includes several error types that reflect other language production difficulties.

^{xxv} In language, semantic pertains to the meaning of the words.

- **Novel words** are those invented by the participant or existing words used in a novel context, e.g. the use of *parst* for column.
- **Phonological errors** involve substituting a word that is phonologically similar to the target, such as using *file* in place of *fire*.
- The **unrelated error** occurs when a participant produces a word that has no relation to the target, e.g. *lever* for *paw*.
- There were no cases of **repetition errors**, which would involve repeating a previous word, unrelated to the target one.
- Additionally, some participants produced **no response errors**, where they did not provide an answer at all (marked as N/A).
- The **grammatical error** is characterised by a semantically related response that is grammatically incorrect, such as using a different word class, e.g. replacing *crying* for *tear*.

Lastly, **Dysfluencies** refer to various disruptions in the speech flow.

- **Circumlocution** occurs when participants use unnecessary extra words to describe a concept, indicating understanding of the concept but difficulties in accessing the target word, as illustrated by saying *dog's foot* instead of *paw*.
- The **planning error** occurs when participants use filled pauses, like *uhm ant* for *ant*, indicating difficulties in word retrieval.
- The **article error** involves the use of articles for the target word violating the task's instructions, .e.g. *a girl* instead of simply *girl*.
- Participants also produced **revision** errors, where they reviewed their responses (e.g., *pepper, salt* for *salt*), and of response **repetition**, where a word was repeated in the same response (e.g., *dinosaur, dinosaur*).
- Finally, **hesitation** errors were made with an unnecessary prolongation in word production, as shown by the expression *nnnnnnut?* for *nut*.

Table 9 below provides a comprehensive summary of errors and disfluencies categories with examples.

Categories	Type	Description	Examples objects	Examples actions
Semantic	superordinate	the participant uses a superordinate word to substitute a more specific word	response/target bug/ant	response/ target cooking/baking
	coordinate	the participant uses a coordinate term to replace the target word	lettuce/celery	sweeping/raking
	subordinate	the participant uses a more specific term (subordinate) to substitute the target word	bungalow/ house	stirring/ cooking
	thematic	the participant uses a term which is thematically related	screwdriver/ drill	eating/serving
Visual	misperception	the participant misinterprets the image because of visual but not semantic misinterpretation	pretzel/ knot	pointing/ teaching
	misinterpretation	the participant misinterprets the image because of a visual misinterpretation, focusing on a small portion of the scene or using a term semantically related to the image but with a different meaning	chin/ woman	bite/ eating
Other	novel words	the participant produces a novel word or uses an existing word with a novel meaning	parst/ column	serm/ serve
	phonological	the participant uses a word which is phonologically related to the target but not semantically	file/ fire	dropping/dripping
	unrelated	the participant produces a word not related to the target	lever/ paw	finnick/ dripping
	repetition	the participant produces an unrelated word as a repetition of an earlier response	no errors of this kind were produced in the sample	no errors of this kind were produced in the sample
	no response	the participant does not produce any response	N/A	N/A
	grammatical	the participant produces a semantically related response but grammatically incorrect (e.g. verb/noun substitution, singular/plural substitution)	crying/tear	prisoners/ arresting
Dysfluencies	circumlocution	the participant uses more words to describe the concept expressed by the target or add unnecessary attributes to the target word	dog's foot/ paw	putting something in the oven / baking
	planning	the participant uses of filled pauses before the word such as ehm, uuu, uhm etc.	uhm ant / ant	aaaa teaching/ teaching
	article	the participant uses the article in front of the word	a girl/ girl	N/A
	revision	the participant changes responses	pepper, salt/ salt	serving, waiting / serving
	repetition	the participant repeats twice the response	dinosaur, dinosaur / dinosaur	Playing, playing / playing
	hesitation	the participant hesitates in producing the word	nnnnut?/ nut	Wwwwhhhistling?/ whistling

Table 9: Classification of the different types of errors in the picture naming task.

This classification is based on a published study which investigated error types in children with Specific Language Impairment (SLI) [115]. The different types have been then adapted to our sample and population and a classification of disfluencies that was not present in the above-mentioned study was added to the different types. Dysfluencies type has been modelled on the definition provided by the software CLAN manual, used for analysis of the picture description task [CLAN.pdf \(talkbank.org\)](http://talkbank.org/CLAN.pdf).

Visual Word Paradigm

Analysis of RTs was coded automatically by PsychoPy with the software capturing mouse clicks for correct responses, which meant the data did not need further processing to undergo statistical analysis. The responses were subsequently assessed for accuracy, revealing a very low rate of errors: fewer than ten in the FXPCs group and fewer than five in the CP. Any incorrect responses were excluded from the analysis. Consequently, only quantitative analysis was conducted for this task, as errors were extremely rare.

Picture description

One of the primary challenges identified in the literature concerning language samples analysis is reliability and reproducibility, as analyses are often highly subjective [243]. Our objective in this analysis is to explore methods for automating the assessment process as much as possible, thus minimising researcher intervention. To achieve this goal, we employed various software tools. The audio recordings were transcribed using the CHAT coding language from the TalkBank repository. The PhD candidate received training through the online resources provided by TalkBank, including the CHAT user manual, and practised the analysis on 16 audio recordings. The transcription process involved meticulously coding and transcribing each linguistic event: accurately assigning speaker roles, appropriately segmenting utterances and aligning them with the corresponding portions of the audio file (Figure 7). Additionally, any extralinguistic material and potential disfluencies or linguistic events were coded following instructions on the CHAT language and CLAN coding material, outlined in the respective user manuals [216]. This was the only phase of the process that required the investigator's involvement: however, the PhD candidate adhered closely the procedures outlined by the TalkBank guidelines [216]. Our analysis specifically focused on the accurate coding of disfluencies, given that the FXPCs population has been documented in the literature to display a more disfluent speech pattern than the control group [77, 79, 80]. Table 10 as shown in the CHAT manual, outlines the various types of disfluencies considered in our analysis.

Stuttering-like disfluencies (SLDs)	Example	Meaning
prolongation	s.poon	the segment “s” is prolonged
broken word	sp oon	there is a pause within the word
blocking	. pillar	there is a block before the word onset
repeated segment	me-medal	the segment “me” segment is repeated
Lengthened repeated segment	memeeedal	The repeated segment is also prolonged
Word repetition	cat/cat	the word “cat” is repeated
Typical Disfluencies (TDs)		
phrase repetition	-this is a- this is a garden	a phrase is repeated
word revision	a woman/mother	revision of the word spoken
phrase revision	they are/the boy is	revision of a phrase spoken
phonological fragment	the wom-the girl	change from “woman” to “girl”
pause	(...)	pause within the utterance or between utterances
filled pause	-um, -aaa, -you know	pauses filled with sound segments

Table 10: Types of codable disfluencies in CHAT.

This table illustrates the different types of disfluencies codable by the software CHAT. A similar table is shown the CHAT talkbank manual to outline the various kinds of disfluencies that can be analysed in the software CLAN. [CHAT.pdf \(talkbank.org\)](https://talkbank.org/CHAT.pdf)

The 121 total audio recordings were then automatically transcribed using the TalkBank approved online ASR service of the rev.ai.com ASR service. The use of this tool requires the batchalign repository, which is a command line program that involves installation of several programs and interaction with the Shell of the machine <https://talkbank.org/info/BA2-usage.pdf> [245]. The transcription output is generated in CHAT format, which helps reduce the time required for transcription. However, each transcribed file still requires manual review and correction. The PhD candidate then verified the accuracy of each transcription by listening to the corresponding audio files and checking for any potential errors. The CHAT transcribing format is required to perform the linguistic analysis on the audio files using a second software developed by TalkBank named is CLAN. For the CHAT output to be analysable by CLAN, the transcription needs to include specific codes, as outlined in the user manual, to mark extralinguistic material, such as gestures or laughter. More importantly, for this study, each potential disfluency, as shown in Table 10, needs to be manually marked using the designated codes. Since automatic speech recognition (ASR) does not consistently detect disfluencies, all relevant codes for each linguistic event had to be manually added to the transcriptions before proceeding with CLAN analysis. We ran several programs on the CLAN commander to perform

the linguistic analysis of the samples. First, we ran the program CHECK which has the aim of controlling for any transcription errors. Secondly, we run the program MOR, FLUCALC and EVAL to obtain a linguistic profile of participants in a picture description task.

MOR: It is a part of the CLAN software, designed for linguistic analysis of spoken language data. MOR specifically focuses on morphological analysis^{xxvi}, which involves analysing the structure of words in transcripts. It automatically tags and assigns parts of speech to words based on pre-defined grammatical rules and lexicons. Even though MOR automatically tags morphology, it may require manual corrections, especially for rare or ambiguous word forms <https://www.talkbank.org/manuals/CLAN.pdf> [245].

FLUCALC: As a fluency and disfluency measurement, FLUCALC analyses transcriptions to identify disfluencies like filled pauses (e.g., "um", "uh"), repetitions, self-corrections, and other non-fluent speech occurrences. It helps researchers quantify the number and types of disfluencies produced in speech. FLUCALC is frequently used in studies involving stuttering, language acquisition in children, or fluency patterns in neurodegenerative conditions. It is useful for both qualitative and quantitative research, providing a detailed breakdown of speech dysfluency patterns.

<https://www.talkbank.org/manuals/CLAN.pdf> [245].

EVAL: is another tool within the CLAN software from TalkBank, designed to assess and evaluate the accuracy of data collected from Aphasic patients and it is used to analyse participants' performance on a discourse task and the output provides several measures of general linguistic performance

<https://www.talkbank.org/manuals/CLAN.pdf> [245].

^{xxvi} Morphology in linguistics is the study of the internal structures of words. Which can vary greatly from one language to another. It also includes grammatical processes such as inflection and derivation. <https://www.britannica.com/topic/morphology-linguistics>

*SP01: &-um I see &-uh a woman and a boy and a girl in a kitchen in a domestic house . %mor: pro:sub I v see det:art a n woman coord and det:art a n boy coord and det:art a n girl prep in det:art a n kitchen prep in det:art a adj domestic n house . %gra: 1 2 SUBJ 2 0 ROOT 3 4 DET 4 2 OBJ 5 4 CONJ 6 7 DET 7 5 COORD 8 4 CONJ 9 10 DET 10 8 COORD 11 10 NCT 12 13 DET 13 11 POBJ 14 10 NCT 15 17 DET 16 17 MOD 17 14 POBJ 18 2 PUNCT *SP01: the elder boy is standing on a stool <with> [/]/ three-legged stool which is tipping over . %mor: det:art the n elder n boy aux be&3S part stand-PRESP prep on det:art a n stool det:num three adj legged n stool pro:rel which aux be&3S part tip-PRESP adv over . %gra: 1 3 DET 2 3 MOD 3 5 SUBJ 4 5 AUX 5 0 ROOT 6 5 JCT 7 8 DET 8 6 POBJ 9 11 QUANT 10 11 MOD 11 8 APP 12 14 LINK 13 14 AUX 14 11 CMOD 15 14 JCT 16 5 PUNCT *SP01: he's reaching into a cookie jar in the cupboard . %mor: pro:sub he~aux be&3S part reach-PRESP prep into det:art a n cookie n jar prep in det:art the n +n cup+n board . %gra: 1 3 SUBJ 2 3 AUX 3 0 ROOT 4 3 JCT 5 7 DET 6 7 MOD 7 4 POBJ 8 3 JCT 9 10 DET 10 8 POBJ 11 3 PUNCT *SP01: the lid of the cookie jars off . %mor: det:art the n lid prep of det:art the n cookie v jar-3S adv off . %gra: 1 2 DET 2 6 SUBJ 3 2 NCT 4 5 DET 5 3 POBJ 6 0 ROOT 7 6 JCT 8 6 PUNCT	*SP01: he's passing a #biscuit to his sister who's standing next to him on the floor next to the stool . %mor: pro:sub he~aux be&3S part pass-PRESP det:art a n biscuit prep to det:poss his n sister pro:rel who~aux be&3S part stand-PRESP adv next prep to pro:obj him prep on det:art the n floor adv next prep to det:art the n stool . %gra: 1 3 SUBJ 2 3 AUX 3 0 ROOT 4 5 DET 5 3 OBJ 6 3 JCT 7 8 DET 8 6 POBJ 9 11 LINK 10 11 AUX 11 8 CMOD 12 11 JCT 13 11 JCT 14 13 POBJ 15 11 JCT 16 17 DET 17 15 POBJ 18 11 JCT 19 11 JCT 20 21 DET 21 19 POBJ 22 3 PUNCT *SP01: &-um the woman is washing up . %mor: det:art the n woman aux be&3S part wash-PRESP adv up . %gra: 1 2 DET 2 4 SUBJ 3 4 AUX 4 0 ROOT 5 4 JCT 6 4 PUNCT *SP01: the sink is overflowing onto the floor . %mor: det:art the n sink aux be&3S over#part flow-PRESP prep onto det:art the n floor . %gra: 1 2 DET 2 4 SUBJ 3 4 AUX 4 0 ROOT 5 4 JCT 6 7 DET 7 5 POBJ 8 4 PUNCT *SP01: she's wiping &-um a plate with a dish cloth adjacent to an open window with a view of the garden . %mor: pro:sub she~aux be&3S part wipe-PRESP det:art a n plate prep with det:art a n dish n cloth adj adjacent prep to det:art a adj open n window prep with det:art a n view prep of det:art the n garden . %gra: 1 3 SUBJ 2 3 AUX 3 0 ROOT 4 5 DET 5 3 OBJ 6 3 JCT 7 9 DET 8 9 MOD 9 6 POBJ 10 9 POSTMOD 11 10 JCT 12 14 DET 13 14 MOD 14 11 POBJ 15 10 JCT 16 17 DET 17 15 POBJ 18 17 NCT 19 20 DET 20 18 POBJ 21 3 PUNCT *SP01: the curtains at the window and on the countertop a plate and #two cups . %mor: det:art the n curtain-PL prep at det:art the n window coord and prep on det:art the n +n counter+n top det:art a n plate coord and det:num two n cup-PL . %gra: 1 2 DET 2 0 INCROOT 3 2 NCT 4 5 DET 5 3 POBJ 6 2 CONJ 7 6 COORD 8 9 DET 9 7 POBJ 10 11 DET 11 7 POBJ 12 11 CONJ 13 14 QUANT 14 12 COORD 15 2 PUNCT
A *SP01: &-um (.) kids trying to get the cookies . %mor: n kid-PL part try-PRESP inf to v get det:art the n cookie-PL . %gra: 1 0 INCROOT 2 1 XMOD 3 4 INF 4 2 COMP 5 6 DET 6 4 OBJ 7 1 PUNCT *SP01: &-uh boy falling from the #chair . %mor: co boy part fall-PRESP prep from det:art the n chair . %gra: 1 2 COM 2 0 ROOT 3 2 JCT 4 5 DET 5 3 POBJ 6 2 PUNCT *SP01: mom's washing dishes . %mor: n mom~aux be&3S part wash-PRESP n dish-PL . %gra: 1 3 SUBJ 2 3 AUX 3 0 ROOT 4 3 OBJ 5 3 PUNCT *SP01: and the water &-um overflow and so leaking on the floor . %mor: coord and det:art the n water over#v flow coord and adv so part leak-PRESP prep on det:art the n floor . %gra: 1 4 LINK 2 3 DET 3 4 SUBJ 4 0 ROOT 5 4 CONJ 6 7 JCT 7 5 COORD 8 7 JCT 9 10 DET 10 8 POBJ 11 4 PUNCT *SP01: &-um #girl is asking for the #cookie . %mor: n girl aux be&3S part ask-PRESP prep for det:art the n cookie . %gra: 1 3 SUBJ 2 3 AUX 3 0 ROOT 4 3 JCT 5 6 DET 6 4 POBJ 7 3 PUNCT *SP01: mom has no reaction for the water (..) for the falling #child . %mor: n mom v have&3S qn no n reaction prep for det:art the n water prep for det:art the part fall-PRESP n child . %gra: 1 2 SUBJ 2 0 ROOT 3 4 QUANT 4 2 OBJ 5 2 JCT 6 7 DET 7 5 POBJ 8 2 JCT 9 11 DET 10 11 MOD 11 8 POBJ 12 2 PUNCT	B

Figure 7: Picture description task's transcriptions in CHAT language.

(A) transcription in CHAT language of a control participant audio recording. (B) transcription in CHAT language of a FXPCs participant audio recording. %mor, %gra= linguistic analysis for morphology and grammar done by the CLAN program "MOR". &-um, &-uh, (.), (..), #, [/]/ = CHAT symbols to mark different types of disfluencies. The control participant's language results richer, and complex with more subordinate sentences, more descriptive and longer utterances compared to the shorter, less detailed and more dysfluent language of the FXPCs participant.

Below a summary table of the different measures considered to analyse the presence of disfluencies from the FLUCALC program.

Disfluency measure	Definition
morUtts	The total number of utterances in the sample
morWords	Total intended words
morsyllables	Total syllables
wordsmn	Number of words
syllablesmn	Number of syllables in the words
Prolongation	% of prolonged segments
Brokenword	% of pauses within words
Block	% of block before word onset
PWR	% part-word repetition
PWRRU	% part-word repetition unit, or iterations. The number of repetitions in a part-word
WWR	% whole word repetition
monoWWR	% monosyllabic repetition
WRRU	% whole word repetitions unit
monoWRRU	% monosyllabic repetitions unit
MeanRU	Mean of repetition units (sum of different repetition unit divided the number of total
Phonologicalfragment	% abandoned word attempts, where the speaker changes word choice.
Phraserepetitions	% of repetitions of entire phrases
Wordrevisions	% of word revisions
Phraserevisions	% of phrase revisions
Pauses	% of utterance-internal unfilled pauses
Filledpauses	% of filled pauses
TD	% of typical disfluencies (sum of phrase repetitions, word revisions, phrase revisions, pause counts, phonological fragments, and filled pauses)
SLD	% stutter-like disfluencies (sum of prolongations, broken words, blocks, PWRs, and monosyllabic WWRs)
TotalSLDTD	% sum of total disfluencies (both typical and stutter like).

Table 11: Definitions of the types of disfluencies analysable in CLAN.

This table offers definition of the different types of disfluencies as explained in the CLAN Talk bank user manual. [CLAN.pdf \(talkbank.org\)](http://CLAN.pdf(talkbank.org))

The second type of linguistic analysis we performed is run by the EVAL command in CLAN which analyses the transcripts and yields analysis of several more general language measures, as outlined previously. The program has been specifically designed for aphasia and dementia; therefore, we believed these measures particularly useful for our sample.

Below a summary table of the different measures considered to analyse the presence of disfluencies from the EVAL program.

Type of language measure	Description
Duration	total time of the sample in hours minutes, seconds.
Total Utts	total utterances.
MLU Utts	number of utterances used to compute MLU.
MLU Words	MLU in words.
MLU Morphemes	MLU in morphemes.
FREQ types	total word types as counted by FREQ.
FREQ tokens	total word tokens as counted by FREQ.
FREQ TTR	type/token ratio
Words/min	words per minute (FREQ tokens/Duration converted to minutes)
Verbs/Utt	verbs per utterance
Density	measure of propositional idea density.
% Nouns	percentage of nouns
% Plurals	percentage of plural tokens
% Verbs	percentage of verbs
% Aux	auxiliaries
% Mod	modals (can, could, shall, should, will, would, may, might, and must)
% 3S	third person singular
% 1S/3S	identical forms for first and third person (e.g., I was, he was)
% Past	Percentage of past tenses
% PastP	past participle
% PresP	present participle
% prep	prepositions
%adv	adverbs
%adj	adjectives
% conj	conjunctions
% det	determiners (includes articles, demonstratives, interrogatives, numbers, and possessives used as determiners)
% pro	pronouns
noun/verb ratio	total # of nouns ÷ total # of verbs (excluding auxiliaries and modals)
open/closed ratio	total # of open class words ÷ total # of closed class words;
#open-class	total # of open class words
#closed-class	total # of closed class words
retracing [/]	number retracing (self-corrections or changes)
repetition [/]	number of repetitions

Table 12 EVAL language measures in CLAN.

This table provides a definition of the language measures analysed by the program EVAL in the CLAN software. CLAN.pdf (talkbank.org)

Statistical data analysis

Picture naming task

To answer our first research question (Is it possible to identify potential differences between FXPCs and a control group through language assessment in FXPCs?), we hypothesised the possibility of finding differences in language assessment between the CP group and the FXPCs group in three different linguistic tasks.

First we investigated this hypothesis in the picture naming in RTs, hypothesising that the FXPCs group would be slower than the CP group particularly in actions, similarly to what has been found in literature on PD. Additionally, we hypothesised that scoring modalities would not yield different results, therefore, to compare group performance we performed a mixed three-way ANOVA including group (CP and FXPCs), word class (actions and object), and modality of analysis (manual and automatic). We also hypothesised that modality of task administration (online and in-person) would influence the performance, therefore we run a second mixed three-way ANOVA including group, word class and the different modality of task administration. We then investigated the same hypothesis in the picture naming task, on potential differences in accuracy running first a mixed three-way ANOVA including group, word class and modality of task administration including all error types, and then we performed another similar mixed three-way ANOVA, this time including all error types but excluding dysfluencies, to have purer measure of actual mistaken answers as disfluencies represent a specific type of deviation from the standard that has been found already in literature on FXPCs therefore we believed it was important to be investigated separately. All the three-way ANOVA's post hoc analysis were corrected for multiple comparison with Benjamini–Hochberg False Discovery Rate (FDR), with a significant threshold of $q < 0.05$. This test controls the proportion of false positives (type I errors) in multiple comparison, it is widely used in studies in language assessments [246] as it represents the perfect balance between minimising the possibility of encountering a type I error and minimise the type II error (false negative), maximising power[247]. Subsequently, we explored the same hypothesis investigating potential different patterns with qualitative analysis across groups, modalities, and word classes. This analysis involved classifying errors into macro categories and then further into subcategories [115], as outlined in Table 9. Percentage of errors was calculated based on the total number of each type of error made by each participant throughout the 60 trials of each word class. The sum of each type of error for the whole group was used to calculate the percentage of errors over the total number of errors made by the group. Statistical analysis was not applied to the qualitative data, as the aim was to understand the various error patterns rather than impose statistical tests on qualitative observations. Results are presented as mean $\pm$ SEM.

Visual Word Paradigm

Subsequently, we investigated the hypothesis of finding differences in linguistic assessment in a language reception task, the visual word paradigm, hypothesising again a poorer performance in actions for the FXPCs group compared to the CP group. First we performed a two-way ANOVA between groups and word class, considering all participants. Then, to factor in the potential effect of modality of task of administration we performed a mixed three-way ANOVA, analysing groups, word class and modality of task of administration. Again post hoc analysis was corrected for multiple comparison with Benjamini–Hochberg False Discovery Rate (FDR). Results are presented as mean $\pm$ SEM.

Picture description

Finally, we investigated potential differences in language assessment to answer the first research question in the picture description task. Here we hypothesised the FXPCs group would display a more disfluent speech pattern compared to the CP group. The task was administered in a consistent way both in-person and online. Given the less structured nature of this task compared to the first two, we did not anticipate major differences between the in-person and the online group. Consequently, to investigate any potential influences between modality we conducted separate ANCOVAs comparing FXPCs and CP, using the modality of task administration (in-person versus online) as a covariate. In cases where the ANCOVA revealed significant differences between modality, subsequent independent t-tests were performed to further investigate these differences. Comparison between groups was investigated in all measures for disfluencies and language described in Table 11, and Table 12. Results are presented as mean $\pm$ SEM.

To be noted that the missing data points in each type of analysis is due to technical issues (the software did not record properly the audio files making them unavailable, or the automatic algorithm was not able to detect onset time). Statistical tests were performed with InvivoStat (version 4.3.), IBM SPSS statistics (version 29.0.1.0 (171)) and Graphpad Prism (version 10.2.3). while to graphically represent the results we used Graphpad Prism (version 10.2.3).

Correlation between tasks

To answer our second research question (what is the nature of language disfluencies patterns in FXPCs and are these related to a primary language deficit?), we correlated disfluency measures from the picture description task (considering the measures where the FXPCs group showed significantly poorer performance compared to the CP group) with latency and accuracy measures from the other tasks.. We hypothesised potential correlation between tasks that would indicate whether disfluencies in a less controlled task, like picture description, are associated with the time needed for word retrieval in tasks tapping directly into the lexical access process. Previous studies failed in finding a link between disfluency measures and deficits in executive function [8], so we aimed to investigate whether the nature of these disfluencies might instead indicate a primary language deficit. Here, we focused exclusively on correlations within the FXPCs group and did not perform the same correlations in the CP group. We employed Spearman rank correlation because at least one of the variables considered for correlation was not normally distributed. These correlations were corrected for multiple comparison with Benjamini–Hochberg False Discovery Rate (FDR), with a significant threshold of $q < 0.05$.

Exploratory correlation analysis

Finally, we performed some additional exploratory analysis as we hypothesised that certain features might affect the results, therefore we conducted several correlations to examine the relationship between task results and independent continuous variables such as age and CGG repeat expansion. We performed Spearman rank correlation because at least one of the variables considered for correlation was not normally distributed. The Spearman rank correlation is a method that measures monotonic associations and is appropriate for non-normally distributed data, ordinal data, and data converted from categorical to ordinal levels [248]. Normal distribution of the data was evaluated using multiple methods, including the D’Agostino & Pearson test, the Shapiro-Wilk test, and the Kolmogorov-Smirnov test. Additionally, normality was visually assessed through a Normal Q-Q plot. These combined approaches ensured a thorough examination of the data's distribution before proceeding with further statistical analyses [249]. Similarly, to analyse correlations involving independent variables represented by categorical data (e.g., gender, medication

use, and history of neurological and neurodevelopmental disorders) and dependent variables (results of the linguistic tasks), we used Spearman's rank correlation. The correlation coefficient range from -1 to +1, where 0 indicates no correlation, +1 signifies a perfect positive monotonic correlation, and -1 indicates a perfect negative monotonic correlation [248]. To be noted that to correlate CGG repeat size we separated allele 1, the allele with the lower repeat number and allele 2 the one with the higher repeat number, and we considered the latter for analysis, as seen in literature [8]. The CGG repeat size was asked to participants together with their medical records, but unfortunately not all participants were able to provide this information, therefore the number of samples correlated with the results was limited. In the in-person group only 7 participants out of 14 were able to provide the number of CGG repeat size, thus it was not ideal to investigate the existence of any potential correlation. Therefore, results of those correlations cannot be considered conclusive.

The exploratory correlation analysis was divided into four distinct steps:

1. Initially, we correlated independent variables, represented as continuous data, such as age and CGG repeat size, with the most relevant results from each task.
2. Next, we correlated the variables concerning the other participants' demographic characteristics classified as categorical data with the results from each task.
3. Subsequently, we performed several within-task correlations to explore potential relationships between latency and accuracy per word in the picture naming task.
4. Finally, we conducted additional Spearman rank correlations involving the psycholinguistic features of each word to determine whether the characteristics of the words selected for each trial correlated with latency and accuracy, as seen in literature [231].

To be noted that the second step required transformation of the categorical variables in ordinal values. We created different categories, and we attributed to each category a number (such as type of medication, type of neurological disorder etc.) applying coding data transformation technique [250], in order to be able to perform correlation analysis through Spearman's rank correlation. For

the medical history of neurological or psychological conditions, each number corresponds to a specific neurological or psychological condition reported by participants. For example, "0" indicates no medical history of neurological conditions, while "1" corresponds to a history of panic attacks, "2" to depression, "3" to FXTAS, and "4" to a combination of depression and anxiety. Other conditions included migraine with Attention-Deficit/Hyperactivity Disorder (ADHD) and depression (5), ADHD with anxiety (6), autism (7), ADHD with anxiety and depression (8), migraines (9), anxiety with depression and migraine (10), migraine with anxiety (11), depression with developmental learning difficulties (12), migraines with FXTAS (13), Transient Ischemic Attack (TIA) (14), migraine with anxiety and ADHD (15), trigeminal neuralgia (16), epilepsy with migraines (17), depression with Post-Traumatic Stress Disorder (PTSD) and anxiety (18), Premenstrual Dysphoric Disorder (PMDD) (18), concussion (19), and migraine with depression (20). For the medications each number represents a particular category of medication. A code of 0 indicates no medication used, while 1 represents steroid use only. A code of 2 corresponds to the use of psychoactive medications, and 3 indicates the use of other types of medications. Code 4 refers to a combination of steroids and other medications, whereas code 5 represents the combined use of psychoactive medications and steroids. Code 6 indicates the use of both psychoactive and other medications, and finally, a code of 7 represents a history of using psychoactive medications, steroids, and other medications together.

2.4 Results

2.4.1 Picture naming: results of latency

To address the first research question in the first linguistic task, we hypothesised that there would be differences between the CP group and the FXPCs group, anticipating slower reaction times in the latter. A mixed three-way ANOVA with a 2x2x2 factorial design was employed (group: CP vs. FXPCS, between subjects; scoring modality: automatic vs. manual, within subject; word class: actions vs. objects, within subject). The analysis supported our hypothesis, revealing a significant main effect of group ($F(1, 222) = 196.5, p < 0.0001$) which indicated different performances between FXPCs and CP. Post hoc pairwise comparisons were conducted to explore differences in RTs across word class, group and modality and p-values were corrected for multiple comparisons as described in

2.3.4. In the automatic scoring the RTs for actions were slower in the FXPCs group compared to the CP group ($q = 0.0183, p = 0.0092$), while in actions in the manual scoring no differences were found ($q = 0.2467, p = 0.1865$). In the object condition the difference between groups was significant in the automatic modality again ($q = 0.0289, p = 0.0155$), but it was not significant in the manual ($q = 0.0701, p = 0.0426$). Although this last effect did not survive FDR correction, the result appears to trend to significance, and the uncorrected significance may suggest a meaningful trend that requires further investigation. Additionally, we anticipated potential differences in word class as seen in literature, and that the different scoring modalities would yield similar results. The analysis supported our hypothesis as we found a main effect of word class ($F(1, 228) = 10.01, p = 0.0018$) indicating a difference between objects and actions, and no effect of scoring modality ($F(1, 228) = 1.942, p = 0.1648$) suggesting no differences between automatic and manual scoring, which confirms both scoring modalities as valid. No interaction effect was found, between word class and group was not significant, ($F(1, 222) = 0.28, p = 0.595$), between word class and scoring modality, ($F(1, 222) = 0.008, p = 0.927$), nor between group and scoring modality, ($F(1, 228) = 0.50, p = 0.482$), or among word class, group, and scoring modality, ($F(1, 222) = 0.89, p = 0.346$). Post hoc pairwise comparisons showed that in the CP group RTs for actions were significantly slower than for objects, with both manual ($q = <0.0001, p = <0.0001$) and automatic scoring ($q = <0.0001, p = <0.0001$). Similarly, in the FXPCs group actions yielded slower RTs than for objects for both manual ($q = 0.0075, p = 0.0032$) and automatic scoring ($q = <0.0001, p = <0.0001$). In the CP group manual scoring didn't yield significantly slower RTs than the automatic in actions ($q = 0.1955, p = 0.1397$), nor in objects ($q = 0.3220, p = 0.2678$). Similarly, in the FXPCs group no effect of scoring modalities was found in the objects ($q = 0.4934, p = 0.4405$) nor in the actions ($q = 0.8423, p = 0.8123$) (Figure 8).

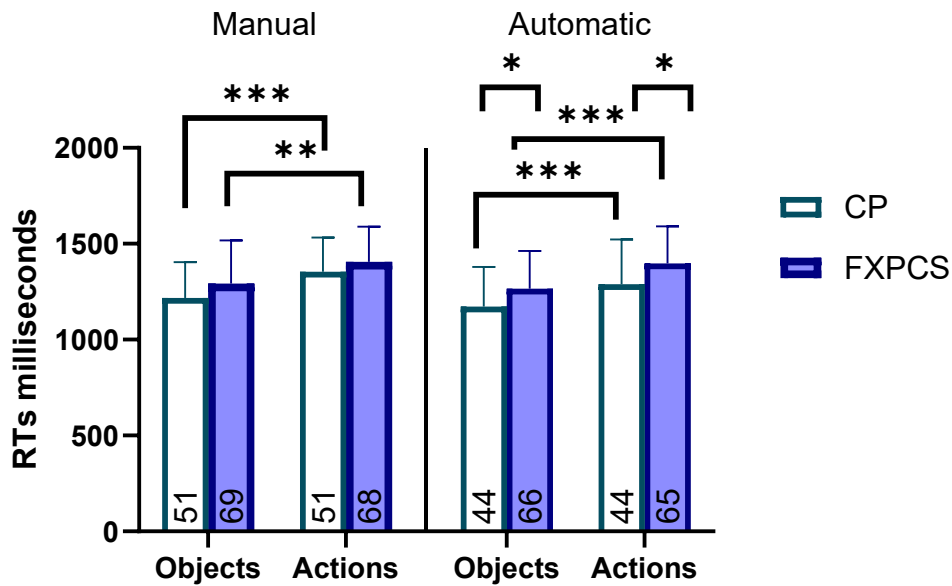


Figure 8. Results of analysis of RTs considering scoring modality..
The automatic scoring modality yielded slower RTs for actions and objects in the FXPCS group compared to the CP group, but not in the manual. However, an uncorrected significant difference is present in the object condition that might indicate a trend where FXPCS are slower in naming objects compared to the CP group. However, it did not survive FDR correction therefore further studies are needed. A significant difference is detected between word class in each group, where actions are named slower than objects in both scoring modalities. No significant difference in scoring modality is found. Data expressed as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

These results partially confirm the main hypothesis of a group difference; however, they were inconsistent between scoring modality therefore no definite conclusions can be drawn based on this task only. Encouragingly, no significant differences between manual and automatic detection methods for voice onset times were observed, therefore this finding may validate both techniques used to detect voice onset time. The different groups effect founds across scoring modalities (significant for actions and objects in automatic modality, and non-significant in the manual modality) may be attributed to variations in voice onset detection due to the different task administration modalities, specifically in-person versus online formats. While detection algorithms for detecting onset times are generally accurate, the dual modality of task administration may limit their effectiveness, especially in controlling for external noises in the online condition. It is uncertain whether the algorithm effectively identifies the actual onset of a word as opposed to consider extraneous factors. In contrast, the manual analysis aimed to mitigate potential disturbances by employing Audacity software to reduce background noise and accurately capture each participant's

voice onset time. This approach aimed to enhance the precision of our measurements and improve the reliability of our findings. Additionally, some data points were missing from the automatic analysis of the RTs, therefore we considered the manual analysis for this specific study to be more accurate, therefore we only used RTs detected with the manual method for subsequent analysis.

In the second phase of the analysis of the picture naming task, we examined the potential impact of task administration modality, anticipating that differences between online and in-person modalities could influence the outcomes. We performed a mixed three-way ANOVAs to compare effects of group (FXPCs vs CP), task administration modality (in-person vs. online) and word class (objects vs actions). Our hypothesis was confirmed as there was a significant effect of modality ($F(1, 116) = 20.82, p < 0.0001$), but no significant group effect ($F(1, 116) = 0.72, p = 0.399$), and the effect of word class was also confirmed ($F(1, 114) = 76.72, p < 0.0001$). All two-way and three-way interactions were not significant: word class $\times$ group was ($F(1, 114) = 0.17, p = 0.679$), word class $\times$ modality was ($F(1, 114) = 0.85, p = 0.360$), group $\times$ modality was ($F(1, 116) = 0.33, p = 0.566$), word class $\times$ group $\times$ modality was ($F(1, 114) = 0.71, p = 0.401$). This indicates that the main effects were consistent across groups and there is no interaction where one factor influences the others in a meaningful way. Post hoc pairwise comparisons were conducted to explore differences in RTs across word class, group and modality and p-values were corrected for multiple comparisons as described in 2.3.4. Task administration modality yielded significant differences where the online modality yielded higher RTs: in objects in the CP group ($q = 0.0154, p = 0.0090$), and in the FXPCs group ($q = 0.0004, p = 0.0002$), in actions in the CP group ($q = 0.0164, p = 0.0110$), and in the FXPCs group ($q = 0.0095, p = 0.0047$). Groups did not produce significant differences: in objects in-person ($q = 0.9616, p = 0.9616$), in objects online ($q = 0.1096, p = 0.0822$), in actions in-person ($q = 0.8819, p = 0.8084$), in actions online ($q = 0.5152, p = 0.4294$). Word class differences confirmed that actions yielded significant slower RTs compared to objects across modalities and groups: in the objects CP in-person vs. actions CP in-person ($q = 0.0004, p = 0.0001$), in the objects CP online vs. actions CP online ($q < 0.0001, p < 0.0001$), in the objects FXPCs in-person vs actions FXPCs in-person ($q = 0.0004, p =$

0.0001), in the objects FXPCs online vs actions FXPCs online ($q < 0.0001$, $p < 0.0001$) (Figure 9).

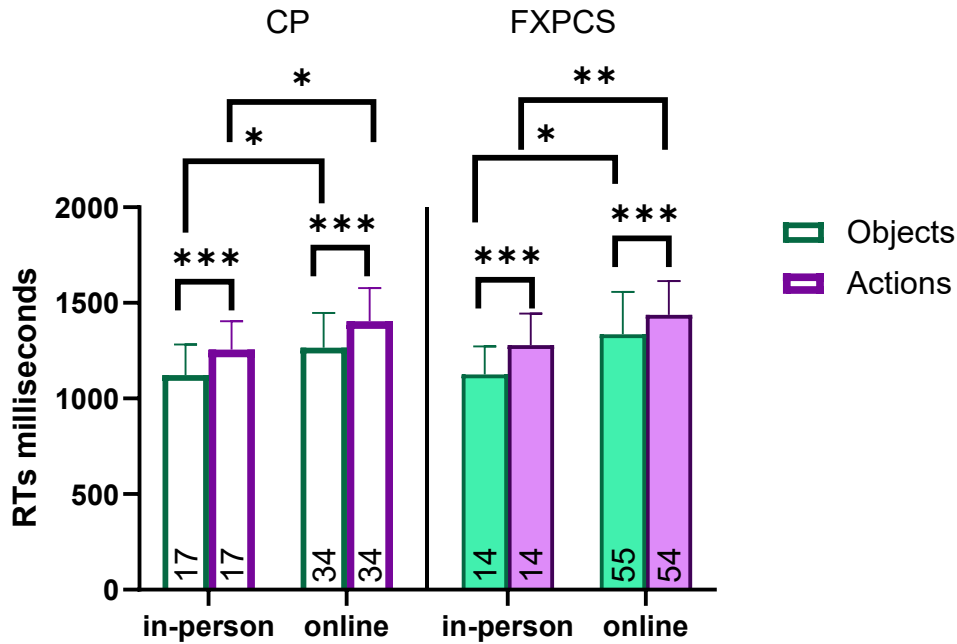


Figure 9: Results of analysis of RTs considering task administration modality.

A significant difference is detected between modality, where in-person participants were faster than the online ones in both groups. No groups differences were found. Significant differences between word class were found across groups, showing that objects are named faster than actions. Data expressed as mean $\pm$ SEM * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.4.2 Picture naming: results of accuracy

The next part of the analysis focused on answering the first research question in the picture naming task focusing on potential differences between groups in their performance in accuracy. We performed a mixed three-way ANOVA to examine the effect of word class (objects vs actions), group (FXPCs vs CP) and modality of task administration (in-person and online) in accuracy, including all types of incorrect responses. We expected to find again differences between groups where the FXPCs group would be less accurate than the CP group, and the analysis confirmed a significant main effect of group ($F(1, 116) = 4.68$, $p = 0.0323$) indicating a different performance between CP and FXPCs. No other significant main effects were present in word class ($F(1, 116) = 0.05$, $p = 0.8221$) indicating no differences between objects and actions in accuracy, or in modality of task administration ($F(1, 116) = 1.30$, $p = 0.2583$). No significant interactions were observed in word class x group ($F(1, 116) = 0.70$, $p = 0.4059$), word class

x modality ($F(1, 116) = 0.13, p = 0.7258$), group x modality ($F(1, 116) = 1.34, p = 0.2491$), or three-way interaction ($F(1, 116) = 0.51, p = 0.4759$). Post hoc pairwise comparison was conducted to explore differences in RTs across word class, group and modality and p-values were adjusted for multiple comparisons, as described in 2.3.4. No statistically significant effects were reported after false discovery rate correction: in modality, in the CP group between actions in-person and online, ($p = 0.0766, q = 0.4597$), and between objects in-person and online ($p = 0.3360, q = 0.7791$), in the FXPCS group between actions in-person and online ($p = 0.8799, q = 0.9008$), and between objects in-person and online ($p = 0.9008, q = 0.9008$), in word class in the CP group in-person ($p = 0.4682, q = 0.7791$), and online ($p = 0.7790, q = 0.9008$), and in the FXPCS group (in-person $p = 0.5759, q = 0.7791$) and online ($p = 0.5843, q = 0.7791$), and finally between CP and FXPCS in the object condition in-person ($p = 0.2518, q = 0.7791$), and online ($p = 0.4472, q = 0.7791$), and in the action condition in-person ($p = 0.0348, q = 0.4182$) and online ($p = 0.3768, q = 0.7791$) (Figure 10).

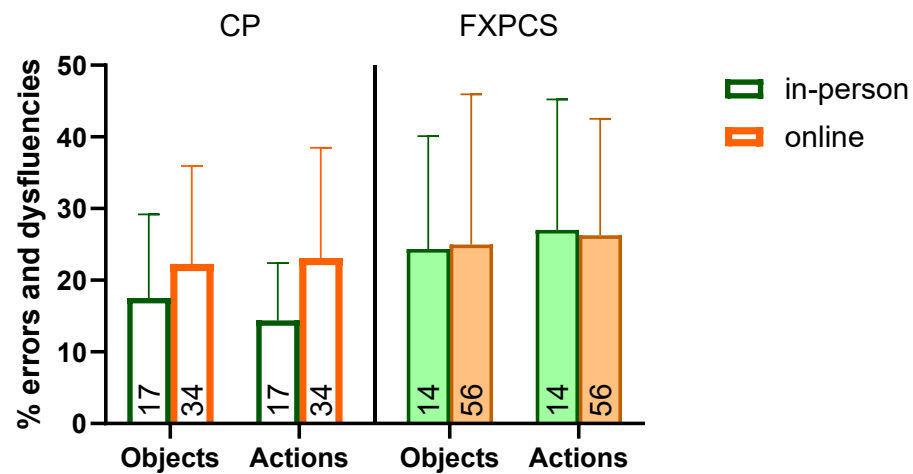


Figure 10: Results of analysis of accuracy considering task administration modality. A significant main effect of group was found that however was not present in the post hoc comparison as none of the comparison were statistically significant. Data expressed as mean $\pm$ SEM.

To further investigate this aspect, in the following analysis we didn't include the error subtype "dysfluencies" as considered inherently different from the other types of errors and verify whether in this case a difference between groups would be more evident. We performed a similar three-way ANOVA including mistaken answers only (excluding disfluencies) to investigate the effect of word class, group, and modality of task administration. The analysis confirmed our

hypothesis and revealed a significant main group effect ($F(1, 116) = 5.30, p = 0.0234$) that did not yield any significant effect in post-hoc comparison, but a significant effect of modality was found in this case ($F(1, 116) = 12.12, p = 0.0008$), and once again no significant effect of word class was found ($F(1, 116) = 0.02, p = 0.8829$), confirming no difference in accuracy between objects and actions. None of the interaction effects were significant: word class x group $F(1, 116) = 0.04, p = 0.8487$, word class x modality $F(1, 116) = 1.18, p = 0.2792$, group x modality $F(1, 116) = 3.23, p = 0.0744$, and the three-way interaction $F(1, 116) = 0.003, p = 0.9545$. Post hoc pairwise comparison was corrected for multiple comparison and revealed two significant effects in the FXPCs group. A modality effect indicates that participants were less accurate in-person compared to the online modality both in the object condition ($p = 0.0005, q = 0.0059$) and in the action condition ($p = 0.0059, q = 0.0357$). This potentially indicate that the in-person modality was less appropriate to assess language abilities in the FXPCs group, potentially due to their higher anxiety levels. This aspect will be further explored in the discussion section 2.5. All other comparisons were not statistically significant after correction, comparing word class in the CP group in-person ($p = 0.6069, q = 0.7600$), online ($p = 0.5003, q = 0.7600$) and in the FXPCs group in-person ($p = 0.7858, q = 0.7858$) and online ($p = 0.2828, q = 0.5656$), comparing modality in the CP group in the objects condition ($p = 0.1562, q = 0.3748$), in the action condition ($p = 0.5668, q = 0.7600$), comparing groups in the object condition in-person ($p = 0.0509, q = 0.1526$), and online ($p = 0.7328, q = 0.7858$), and in the action condition in-person ($p = 0.0356, q = 0.1422$), and online ($p = 0.6334, q = 0.7600$) (Figure 11).

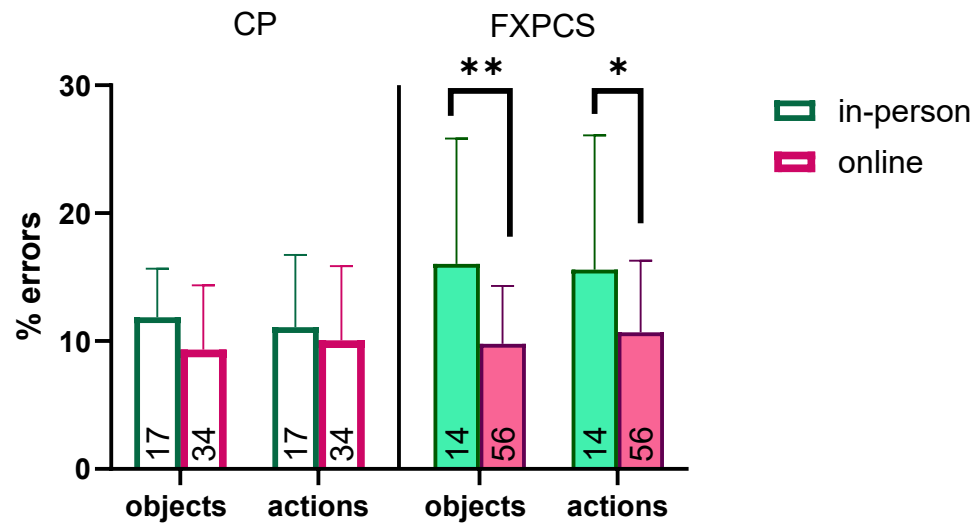


Figure 11: Results of analysis of accuracy considering modality, including mistaken answers only.

The analysis shows a main group and modality effect. The latter indicates in post hoc comparison that the FXPCS group was less accurate in-person compared to the online modality both in objects and in actions. Data expressed as mean $\pm$ SEM * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.4.3 Picture naming: results of qualitative analysis

We further investigated differences between groups in accuracy performing qualitative analysis on the errors to investigate the presence of different error patterns. Because of the differences found between groups, we hypothesised potential different patterns, between word class (objects and actions), and between task administration modality (online and in-person).

Different types of errors

In contrast with our expectation, the qualitative analysis of error types in both object and action conditions, across online and in-person modalities, revealed similar patterns for the FXPCS and CP groups. Disfluencies accounted for the highest proportion of errors in all scenarios (50% for the CP group and 57% for the FXPCS group in the object condition; 57% for the CP group and 60% for the FXPCS group in the action condition). A different pattern was found instead in word class in the second most frequent error type varied by condition but remained consistent across groups: semantic errors ranked second in the object condition, while visual errors took the second spot in the action condition. These were followed by other error types (Figure 12).

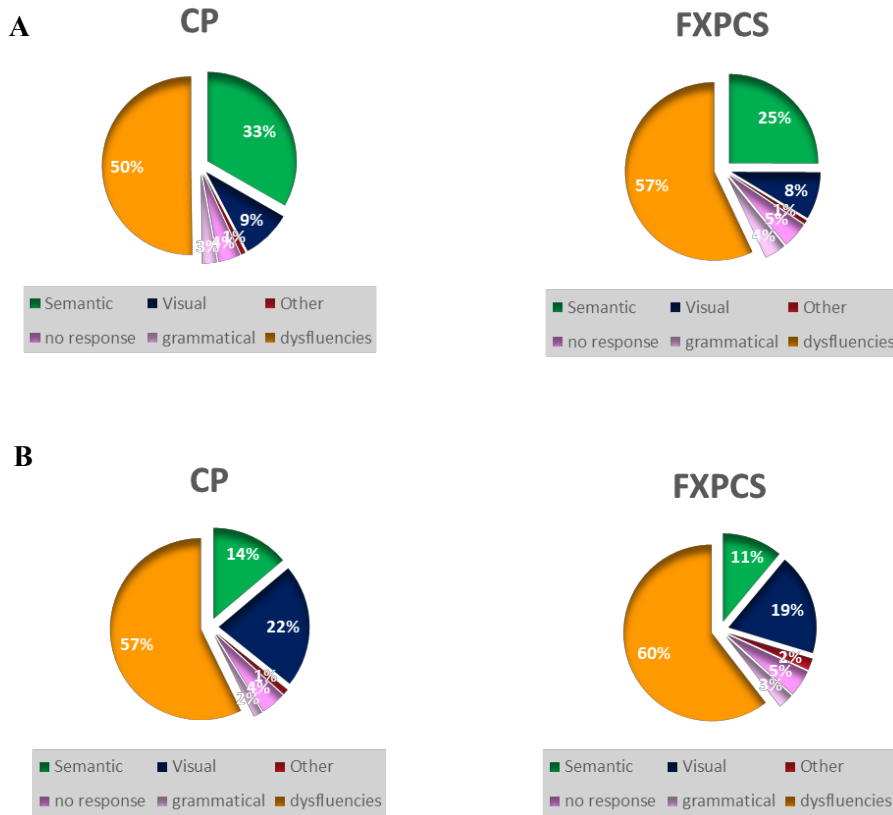


Figure 12: Percentage of errors classified by macro categories of error types for both FXPCs and CP.

(A) In the object condition the highest percentage is represented by dysfluencies followed by semantic type and visual type of errors.

(B) In the action condition the highest percentage is again represented by the dysfluencies, but the second highest this time is represented by visual type of errors, followed by the semantic type.

Errors percentage was calculated based on the total number of each type of error that each participant made throughout the 60 trials, and then the sum of each type of error for all the participants was used to calculate percentage over the total number of errors made by the group.

We further analysed the types of errors by classifying them in subtypes (Table 9). Here a different pattern emerges between groups, as the main observation concerns the dysfluencies were the FXPCs group seems to perform poorly compared to the CP group in the article and circumlocution subtype across word class. While the CP group seemed to have more difficulties in the planning subtype. This pattern has been found in studies analysing similar tasks in PD, potentially indicating difficulties in accessing the target word in the semantic memory, and will be further discussed in the discussion section 2.5 (Figure 13).

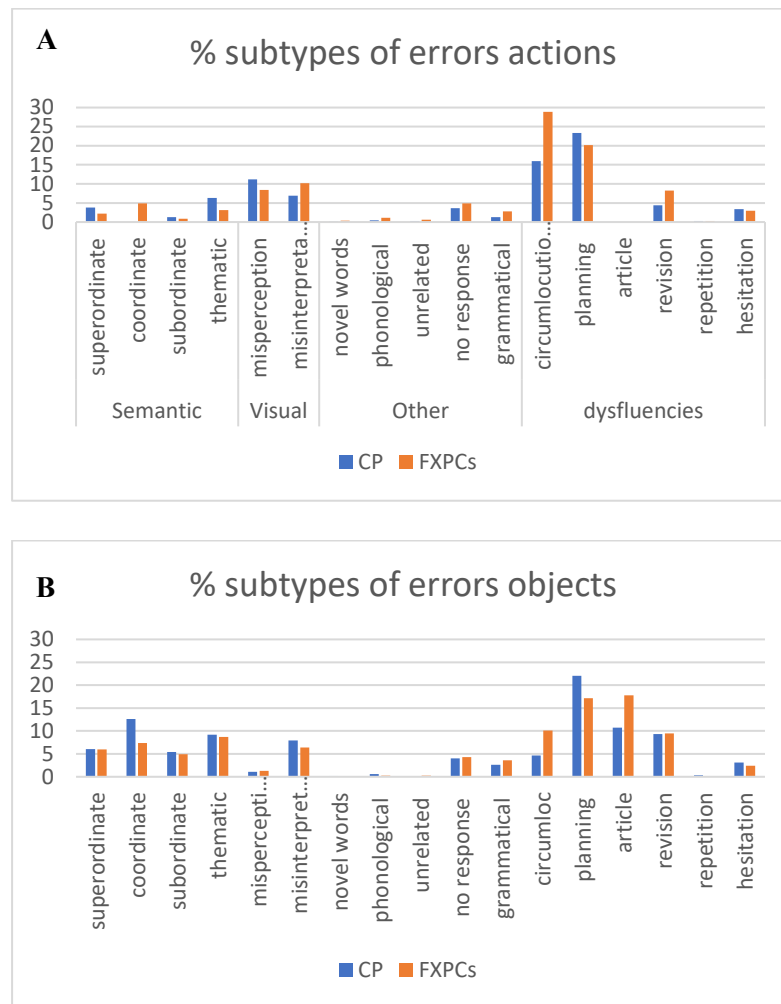


Figure 13: Comparison of subtypes of errors between CP and FXPCs.

(A) Differences in the subtypes of errors between the CP and FXPCs groups in the object condition reveal distinct patterns. The FXPCs group exhibited a higher proportion of circumlocution-type disfluencies, whereas the CP group displayed more planning-related errors. (B) In the action condition, a similar trend emerged: the FXPCs group recorded higher percentages of circumlocution disfluencies, while the CP group showed a greater prevalence of planning errors.

Online

We then compared percentage of errors separating modality of task administration to investigate whether a different pattern emerged for online and in-person participants (Figure 14). The overall pattern of errors is confirmed in the online modality, with the disfluencies subtype representing the highest percentage of errors across word class, followed by semantic type in the object condition and visual type in the action condition.

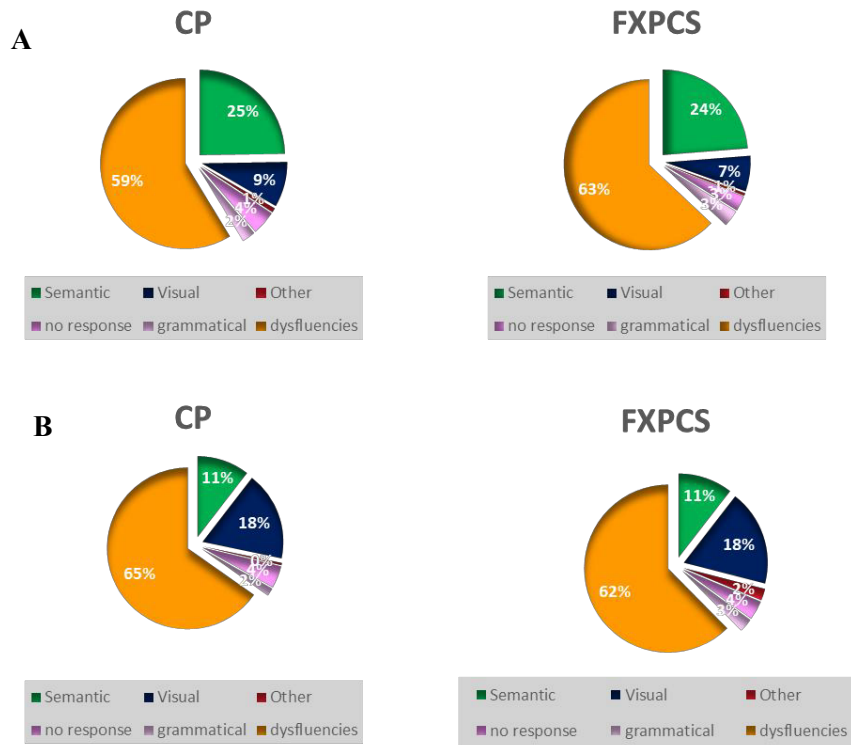


Figure 14: Percentage of errors classified by macro categories of error type in the FXPCS and CP group online.

(A) In the object condition the highest percentage of errors is represented by the dysfluencies type, while the second highest is represented by the semantic type of errors.
 (B) In the action condition the highest percentage is again represented by the dysfluencies type while the second highest portion is represented by the visual type.

As shown in Figure 15 no major differences with the analysis of the two modalities combined was found in the subtypes of errors comparison.

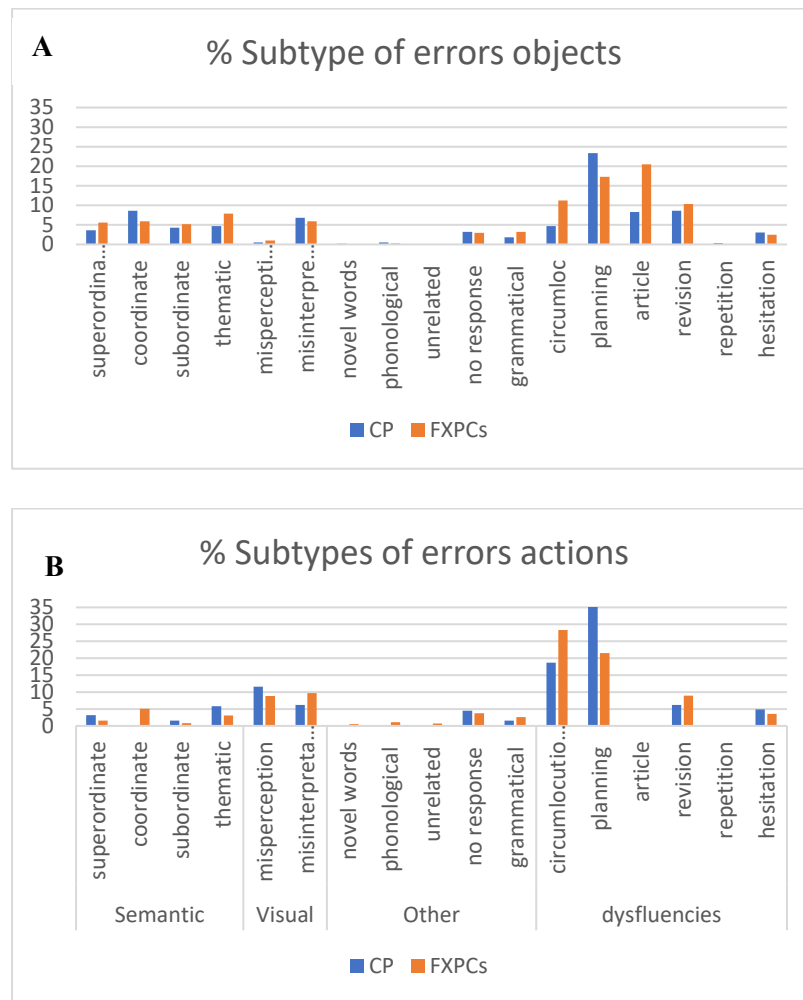


Figure 15: Comparison of subtypes of errors between CP and FXPCs online.

(A) In the object condition a different pattern is present in the dysfluencies subtype, where the circumlocutions subtype represented the highest percentage of errors for the FXPCs group and the planning subtype for the CP group. (B) The same pattern is present in the action condition where again more circumlocutions were produced by the FXPCs group and more planning errors by the CP group..

In-person

The analysis of the in-person group revealed distinct patterns. In the object condition, no noticeable pattern differences were observed between the groups. However, compared to the online group, the CP group exhibited a higher proportion of semantic errors, exceeding 50% in the CP group, followed by disfluencies, visual errors, no response, grammatical errors, and other categories. In the action condition, the FXPCs group maintained a consistent pattern similar to the online modality, whereas the CP group demonstrated nearly equal proportions of visual and disfluencies errors, followed by semantic and other types. These findings suggest that the FXPCs group consistently exhibited a high percentage of disfluencies across both modality and condition, with semantic

errors taking precedence in the object condition. Conversely, the CP group demonstrated variability, with other types of errors potentially influenced by the task administration modality and word classification (Figure 16).

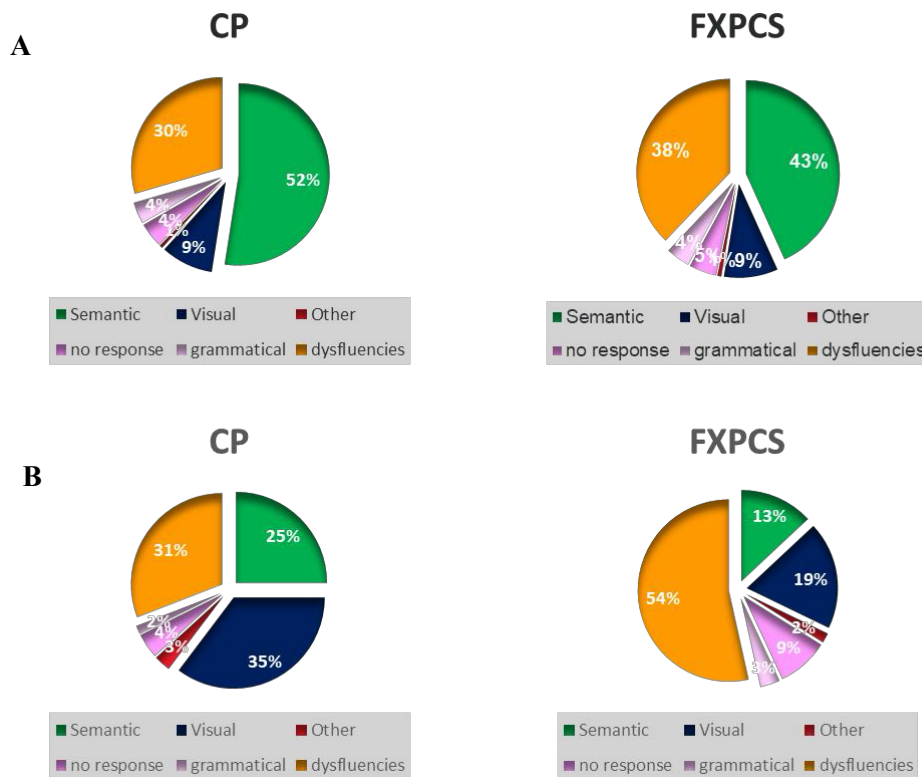


Figure 16: Percentage of errors classified by macro categories of error types in-person.

(A) In the object condition the semantic type of errors is the highest percentage, followed by dysfluencies and visual type. (B) In the action condition there is a difference between FXPCS where again the dysfluency type represents the highest percentage, while in the CP group the visual and dysfluency type represent a similar portion of the total errors, followed by the semantic type.

When breaking down the subtypes of errors, the CP group's performance was poorer in all subtypes of semantic errors in the object condition, and in the visual subtype in the action condition (Figure 17). Whereas the FXPCS group's performance in general was poorer in the dysfluencies type of errors both in objects and in actions, particularly showing poorer performance in the planning, circumlocution, and hesitation subtypes.

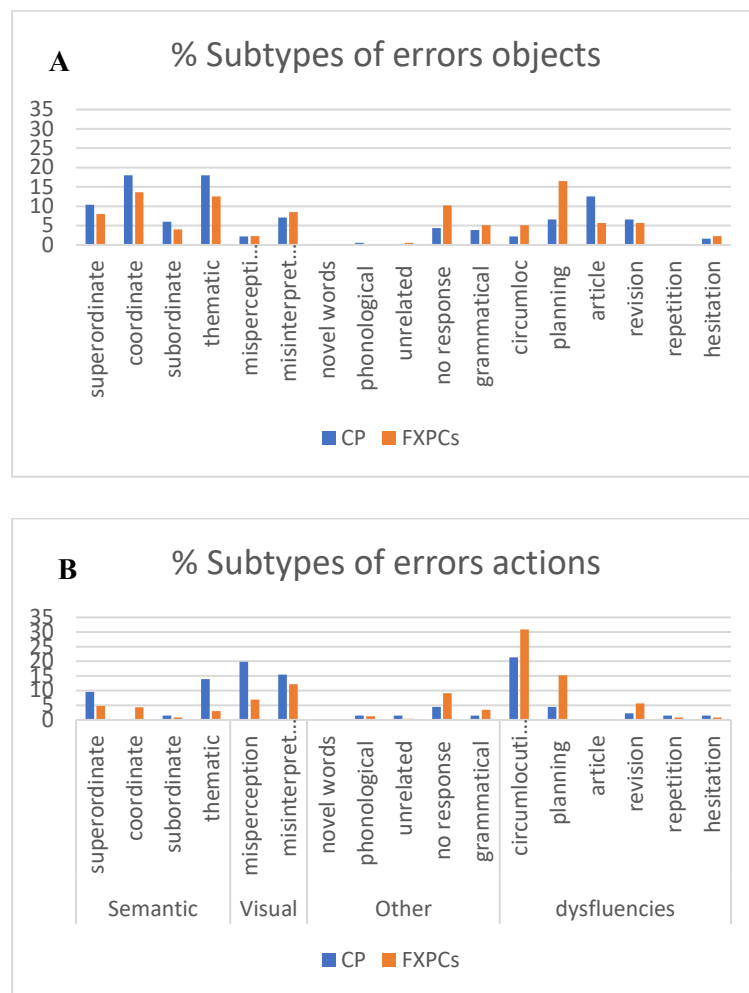


Figure 17: Comparison of subtypes of errors between CP and FXPCs in-person.
 (A) In the object condition the presence of a higher percentage of all subtypes of semantic errors in the CP compared to the FXPCs is noteworthy, while a similar subtype pattern to the online modality is present in the dysfluencies subtype, except for a higher percentage of planning subtype in the FXPCs group. (B) in the action condition circumlocutions and planning subtypes still represent the highest percentage of errors in the FXPCs group, while the CP group shows higher percentages of both the visual types of errors.

In the next figure we show a visual representation of the different subtypes of errors across modalities. In the object condition of the CP group a very evident difference is present in the disfluencies where the planning subtype of error is much more present in the online group than the in-person one, and the in-person group had higher percentages of all semantic subtypes. In the FXPCs group again the in-person participants seem to have more difficulties with the semantic errors, the visual, omissions and grammatical subtype, while in the disfluencies the online group seems to perform poorer in most of the subtypes. In the action condition in the CP group the much higher percentage of planning subtype of dysfluencies in the online group stands out compared to in-person group, while the latter had a poorer performance in the visual and semantic subtypes. Whereas

in the FXPCs group there aren't major differences in error patterns between online and in-person, with slightly higher percentage of visual subtypes for the in-person and slightly highest percentage of circumlocutions subtypes (Figure 18).

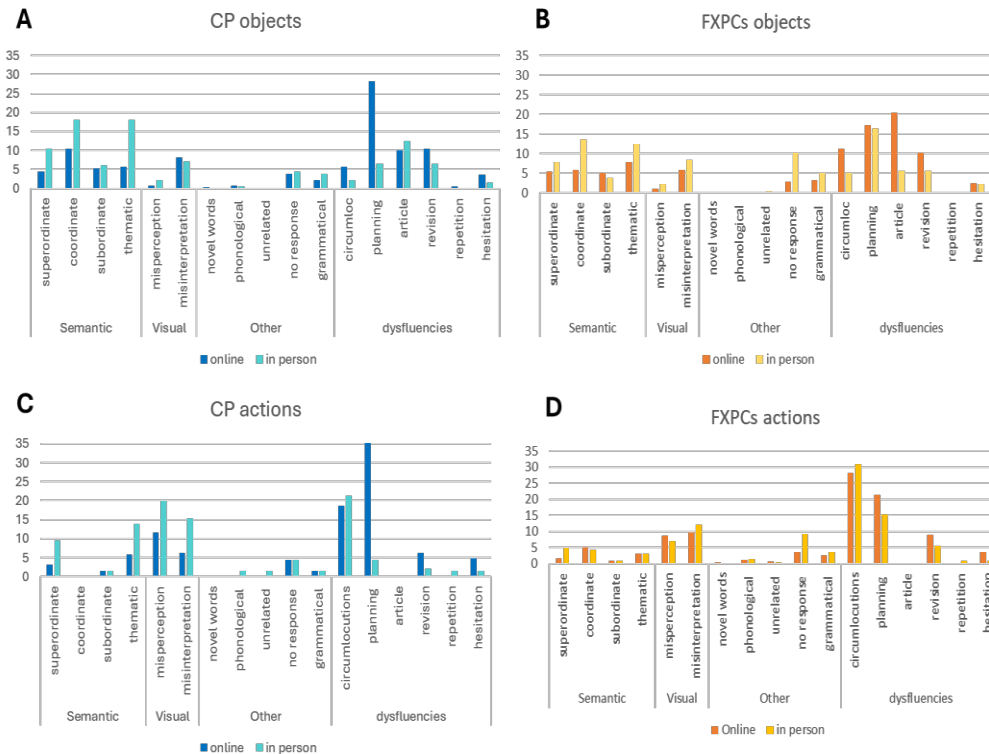


Figure 18: Comparison of subtypes of errors between testing modality, online and in-person.

(A) In the object condition in the CP group the main difference is represented by the highest percentage of planning subtypes in the online modality. (B) In the object condition of the FXPCs group the main differences are seen in the semantic subtypes where the in-person group showed higher percentages of mistakes, while the online group showed higher percentages in the dysfluencies. (C) In the action condition in the CP group again the planning subtype is more present in the online condition, while in this case the visual subtypes are more represented in the in-person group. (D) In the FXPCs group in the action condition, no major differences are present between modalities.

2.4.4 Visual Word Paradigm: results of latency

We then addressed the first research question in the second linguistic task. We hypothesised again that the FXPCs group would be slower in word retrieval compared to the CP group, particularly in actions. We first performed a two-way ANOVA including data of combined modalities (in-person and online) to investigate effect of group and word class. The analysis confirmed the hypothesis of a difference between groups as it revealed a significant main effect of group

($F(1, 234) = 7.73, p = 0.0059$). However, post hoc pairwise comparison that was corrected for multiple comparison shows that there was no difference between groups in both objects ($q = 0.0546, p = 0.0455$), and actions ($q = 0.0559, p = 0.0559$). While this effect was not statistically significant after correction, it might indicate a potential trend where FXPCs are slower compared to CP in objects as the values are trending to significance, but further studies are needed to confirm this. The effect of word class found in the latency analysis of the picture naming is confirmed here ($F(1, 234) = 119.4, p < 0.0001$). Additionally, post hoc pairwise comparison corrected for multiple comparison revealed significant effects in word class where participants were significantly faster in objects compared to actions both in the CP group ($q < 0.0001, p < 0.0001$), and in the FXPCs group ($q < 0.0001, p < 0.0001$) (Figure 19).

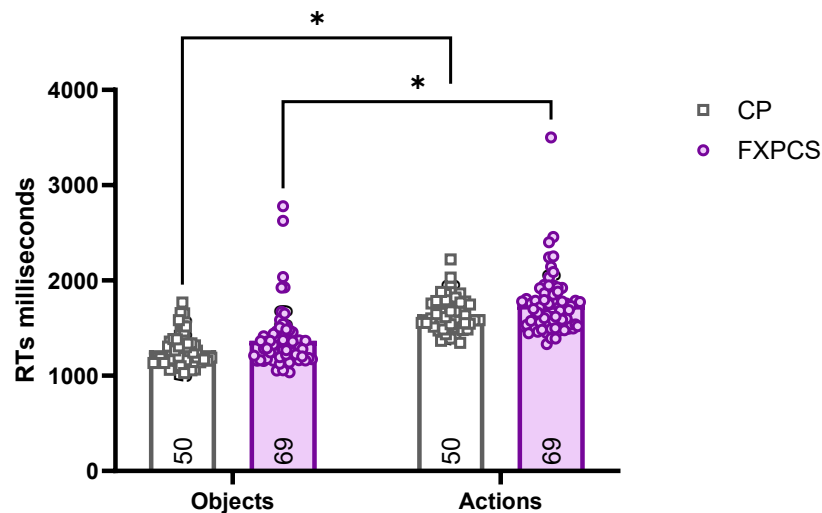


Figure 19: Results of analysis of RTs in the VWP task combining task administration modalities.

A main group effect is present; however no significant differences are present in post hoc comparison. A difference in word class is also present showing that actions elicited slower RTs compared to objects across groups. $*p < 0.05$.

To explore the influence of modality of task administration in this second task, we performed a mixed factorial three-way ANOVA investigating modality of task administration (in-person, online), group (CP, FXPCs) and word class (objects, actions). We expected to find differences between modalities again. The analysis confirmed the hypothesis as it revealed a significant effect of modality ($F(1, 115) = 10.36, p = 0.0017$), while the group effect did not reach significance ($F(1, 115) = 2.12, p = 0.1480$) and also confirmed the effect word class ($F(1,$

115) = 257.0, $p < 0.0001$),. None of the interaction effects was significant, modality x group ($F(1, 115) = 0.06$, $p = 0.8148$), modality x word class ($F(1, 115) = 0.28$, $p = 0.5947$), group x word class ($F(1, 115) = 0.33$, $p = 0.5656$), and the three-way interaction ($F(1, 115) = 0.77$, $p = 0.3812$). Post hoc pairwise comparison was corrected for multiple comparison and revealed that in modality of task administration only in the FXPCs group in the action condition RTs were significantly higher for the online condition compared to the in-person one ($p = 0.0086$, $q = 0.0205$). The other modality of task administration comparisons did not survive correction, in the CP group in the object ($p = 0.0464$, $q = 0.0928$), and in the action condition ($p = 0.0736$, $q = 0.1104$), and the FXPCs group in the object condition ($p = 0.0728$, $q = 0.1104$). No group effect was found after correction, in the in-person modality in objects ($p = 0.3171$, $q = 0.3459$), actions ($p = 0.7749$, $q = 0.7749$), and in the online modality in objects ($p = 0.1715$, $q = 0.2058$) and actions ($p = 0.1063$, $q = 0.1417$). Significant differences of word class were found where participants were faster in objects than actions, in-person in the CP group ($p < 0.0001$, $q < 0.0001$), and in the FXPCs group ($p < 0.0001$, $q < 0.0001$), and online in the CP group ($p < 0.0001$, $q < 0.0001$) and in the FXPCs group ($p < 0.0001$, $q < 0.0001$). (Figure 20).

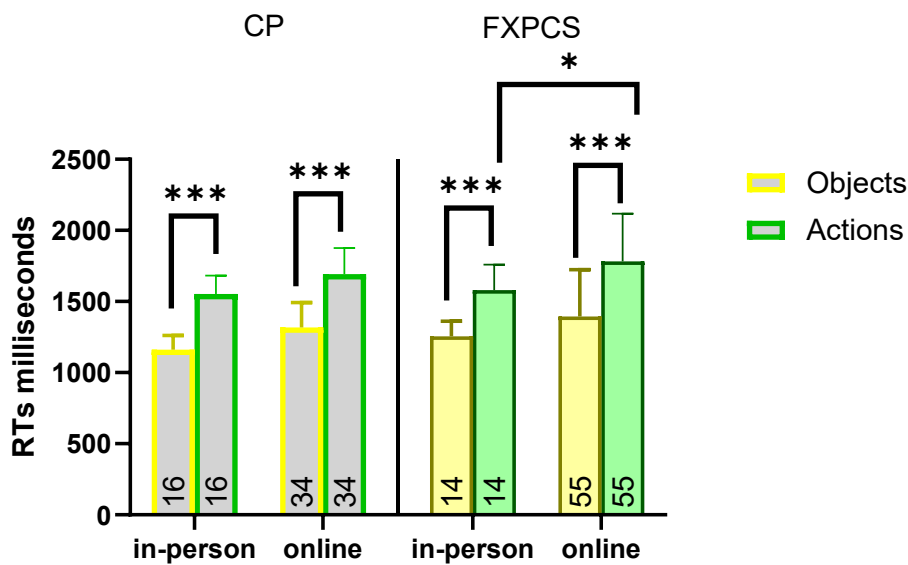


Figure 20: Results of analysis of RTs in the VWP task considering task administration modality.

Task administration modality was different only in the FXPCs group in the action condition, showing that online participants were slower compared to the in-person one. A word class difference is also present, showing that actions elicited slower RTs compared to objects. * $p < 0.05$, *** $p < 0.001$

2.4.5 Picture description: results

Finally, we investigated the third part of our first research question, assessing the presence of potential differences between groups in the picture description task, expecting to find a more disfluent profile in the FXPCs group compared to the CP group. As outlined in the analysis section 2.3.4, since no major differences were expected between modalities, modality was treated as a covariate, as it could influence the variables of interest. ANCOVAs were performed using InvivoStat software. To ensure consistency with the graphical representations used in the rest of the analysis, we employed GraphPad Prism, which does not support ANCOVAs. Consequently, we performed independent t-tests for graphical comparisons when the data met parametric assumptions, and Mann-Whitney tests when they did not. As hypothesised, The ANCOVAs performed to compare the disfluencies measures (FLUCALC) seen in Table 11 did not produce a modality effect in any of the 24 disfluencies variables analysed, confirming that the different testing modality did not affect results in this specific task and only in two of the general language measures (EVAL), the percentage of nouns ($F(1, 108) = 6.45, P = 0.0125$) and the percentage of pronouns ($F(1, 108) = 8.72, P = 0.0039$). The ANCOVA analysis showed a group effect in the following linguistic variables confirming our hypothesis of a difference between groups: the block ($F(1, 115) = 11.50, P = 0.0010$), the word revisions ($F(1, 108) = 5.44, P = 0.0215$), phrase revisions ($F(1, 108) = 6.97, P = 0.0095$), pauses ($F(1, 108) = 6.29, P = 0.0136$), SLD ($F(1, 108) = 7.22, P = 0.0084$) and total SLDTD ($F(1, 108) = 4.74, P = 0.0316$), type of disfluencies and in the following general language measures: the percentage of adjectives ($F(1, 108) = 6.37, P = 0.0130$), and the number of retracing ($F(1, 108) = 10.45, P = 0.0016$). The ANCOVA analysis also showed a non-significant result that was trending to significance in the percentage of prepositions ($F(1, 108) = 2.98, P = 0.0870$), where the CP group produced more prepositions than the FXPCs group. However, it lacked to reach statistical significance, hence any interpretation is speculative. The further comparison were performed for visual representation between groups with the variable of interest, through independent student t-tests or Mann-Whitney tests showed that the FXPCs resulted in a higher percentage of disfluencies compared to the CP group, in the percentage of block type of disfluencies ($P = 0.0018$), in the percentage of word revisions type ($P = 0.0244$), in the percentage of phrase revisions type ($P = 0.0079$), in the percentage of total SLDTD ($P = 0.0428$). It

additionally showed that the CP group produced a significantly higher percentage of adjectives than the FXPCs ($P=0.0071$) while the FXPCs group produced a higher number of retracing ($P=0.0012$), and the CP group produced a non-significantly higher number of prepositions ($P=0.0654$) (Figure 21). The higher number of disfluencies in the FXPCs group and the higher number of adjectives in the CP group potentially indicates that the CP group showed a linguistically richer speech, while the FXPCs group produced a more hesitant and less structured speech.

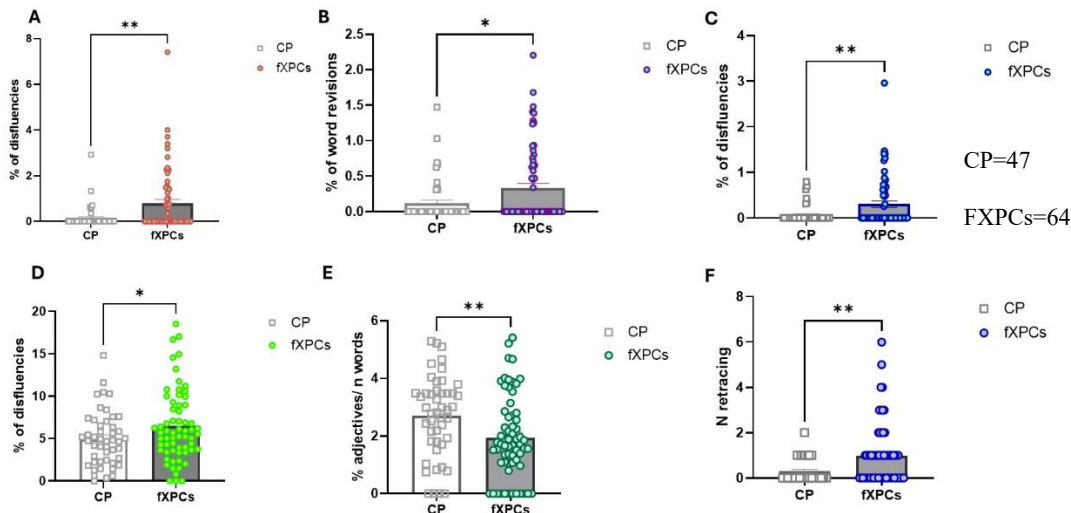


Figure 21: Comparison between groups in the disfluencies and general language measures in the picture description task.

(A) Block type of disfluencies: the two groups did not meet the criteria for parametric *t*-test analysis, therefore Mann-Whitney test was run instead. A significant difference was found where FXPCs produced more Blocks than CP. (B) Wordrevision: it was not possible to perform the *t*-test as the data did not meet the criteria for parametric analysis. The Mann-Whitney test was run instead, and a significant difference was found where FXPCs produced more Wordrevisions than CP. (C) Phraserevisions: it was not possible to perform the *t*-test as the data did not meet the criteria for parametric analysis. The Mann-Whitney test was run instead, and a significant difference was found where FXPCs produced more Phraserevisions than CP. (D) Total SLDT: The unpaired *t*-test revealed a significant difference between FXPCs and CP in the percentage of total SLDT type of disfluency, where FXPCs showed a higher percentage of disfluencies compared than the CP group. Data expressed as mean $\pm$ SEM * $p<0.05$, ** $p<0.01$.

Other comparisons were not significant such as the percentage of pauses ($P=0.1018$) and in the percentage of SLD ($P=0.0597$) and the CP group produced a non-significantly higher number of prepositions ($P=0.0654$).

2.4.6 Correlation among linguistic tasks

To answer our second research question, we performed a series of Spearman Rank correlations to correlate language disfluencies measures in the picture

description task to latency and accuracy in the other tasks. In the picture description task, we only considered those measures where the FXPCs group revealed a particularly disfluent pattern compared to the CP group. Because we did not find major differences in this task between in-person and online participants we did not separate the analysis per modality of task administration in this case. Additionally, we only performed the correlation with the FXPCs group, because the CP group did not show a disfluent language pattern compared to the FXPCs group. These correlations were calculated to assess the strength and direction of associations between key variables of interest, providing insights into potential patterns and underlying factors that might correlate with the results. The Spearman Rank correlation between latency in the object condition of the picture naming in the FXPCs group and the disfluency measures in the picture description task revealed a significant positive monotonic correlation with the disfluency measure Total SLDTD ($r(61) = 0.4103$, $p = 0.0008$). This correlation survived FDR correction for multiple comparison $q=0.0040$, indicating that as the disfluencies measure increased the time to retrieve objects increase as well. The retracing number measure showed also a significant correlation ($r(61) = 0.2644$, $p = 0.0363$), however the p-value did not survive FDR correction with the Benjamini-Hochberg procedure ($q=0.0908$). The analysis did not yield any significant correlation with the Block ($r(60) = 0.09184$, $p = 0.4777$), Wordrevisions ($r(61) = 0.2359$, $p = 0.0627$), Phraserevision ($r(61) = 0.2091$, $p = 0.1000$) types of disfluencies. The correlation between latency in the action condition of the picture naming in the FXPCs group and the disfluency measures in the picture description task revealed a significant positive monotonic correlation with the disfluency measure Wordrevisions ($r(61) = 0.3675$, $p = 0.0033$, $q=0.0083$), Total SLDTD ($r(60) = 0.4022$, $p = 0.0012$, $q=0.0060$) and the retracing number ($r(60) = 0.3376$, $p = 0.0073$, $q=0.0122$). The analysis did not yield any significant correlation with the Block ($r(59) = 0.02694$, $p = 0.8367$), Phraserevision ($r(60) = 0.2136$, $p = 0.0955$) types of dysfluencies. The correlation between accuracy in the object condition of the picture naming in the FXPCs group and the disfluency measures in the picture description task did not yield any significant correlation with the disfluency measure Block ($r(61) = -0.05331$, $p = 0.6782$), Wordrevisions ($r(62) = 0.03861$, $p = 0.7620$), Phraserevision ($r(62) = 0.1407$, $p = 0.2674$), Total SLDTD ($r(62) = 0.2294$, $p = 0.0682$) and the Retracing number

($r(62) = 0.1134, p = 0.3722$) types of disfluencies, showing potentially no relationship between accuracy performance in lexical access and disfluencies measures. The correlation between accuracy in the action condition of the picture naming in the FXPCs group and the disfluency measures in the picture description task, did not reveal any significant correlation with the disfluency measure Block ($r(61) = -0.02258, p = 0.8606$), Wordrevisions ($r(62) = -0.07042, p = 0.5803$), Phraserevision ($r(62) = 0.05966, p = 0.6396$), Total SLDTD ($r(62) = 0.1302, p = 0.3052$) and the retracing number ($r(62) = 0.04699, p = 0.7123$), showing potentially no relationship between accuracy performance in lexical access and disfluencies measures. The correlation between latency in the object condition of the Visual Word Paradigm in the FXPCs group and the disfluency measures in the picture description task, did not reveal any significant correlation with the disfluency measure Block ($r(60) = -0.0007462, p = 0.9954$), Wordrevisions ($r(61) = 0.1939, p = 0.1279$), Phraserevision ($r(61) = 0.1387, p = 0.2783$), Total SLD ($r(61) = 0.06841, p = 0.5942$) and the retracing number ($r(61) = 0.1664, p = 0.1925$), showing potentially no relationship between the performance in the language reception task and disfluencies measures. Finally, the correlation between latency in the action condition of the Visual Word Paradigm in the FXPCs group and the disfluency measures in the picture description task, did not yield any significant correlation with the disfluency measure Block ($r(60) = 0.1173, p = 0.3640$), Phraserevision ($r(61) = -0.008718, p = 0.9459$), and the Retracing number ($r(61) = 0.1094, p = 0.3934$). However, it did show a significant monotonic positive correlation with Total SLD ($r(61) = 0.3013, p = 0.0164, q=0.0820$) that did not pass FDR correction, and a weak positive correlation with Wordrevisions ($r(61) = 0.2419, p = 0.0561$), that however was not significant (Figure 22). Overall, we believe the presence of some correlations show potential to be further investigated and indicates a relationship between performance in tasks tapping directly into lexical access and disfluencies measures, where the percentage of disfluencies increases so does the time to retrieve action words.

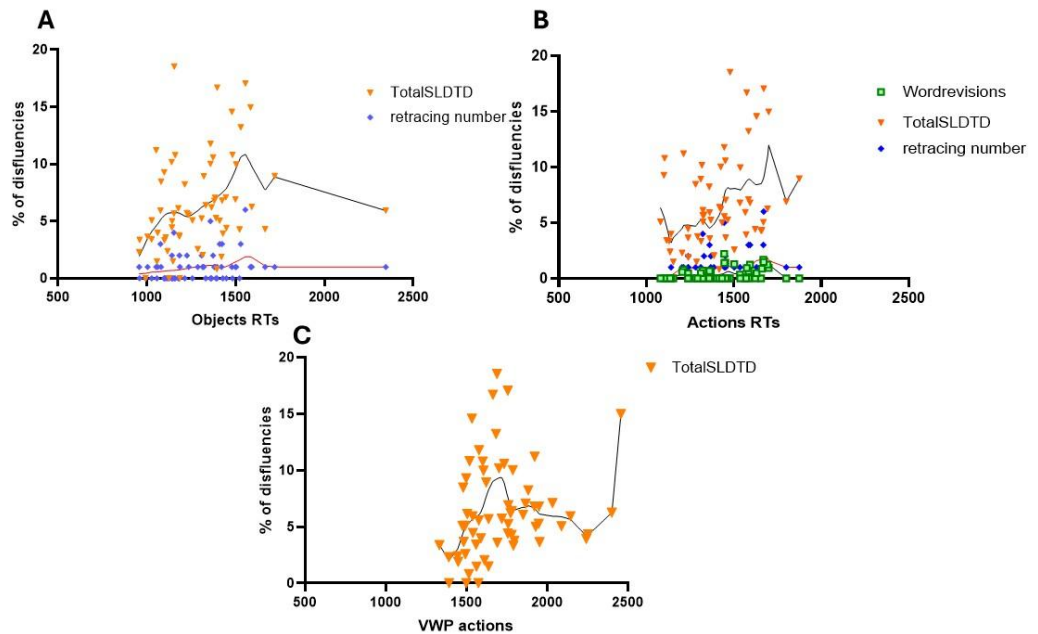


Figure 22: Correlations between percentage of disfluencies in the picture description task and RTs in the picture naming and VWP task.

(A) In the picture naming task, the two disfluencies measure Total SLDTD, and the retracing number showed a correlation with the Objects RTs, however the latter was not significant. (B) In the picture naming task, the three disfluencies measure Wordrevisions, Total SLDTD and the Retracing number showed a correlation with the Actions RTs. (C) In the VWP the disfluency measure TotalSLDTD showed a correlation with the actions RTS, that however was not significant.

2.4.7 Exploratory correlation analysis

To finalise analysis of linguistic task we subsequently run a series of exploratory analysis to investigate potential correlations with factors that might have influenced the results, particularly demographic features.

Correlation with age

Picture naming task (RTs)

The first independent variable we considered is the age and we correlated it with the different results in the picture naming task. No correlation was found in the FXPCs group between age and the picture naming task results in objects RTS $r(67) = 0.1516$, $p = 0.2137$, and in the actions RTs $r(66) = 0.1916$, $p = 0.1176$ while a moderate correlation with age was identified in the CP group's responses, both in the objects RTs $r(49) = 0.2717$, $p = 0.0538$ and in the actions RTs $r(49) = 0.3386$, $p = 0.0151$ (Figure 23).

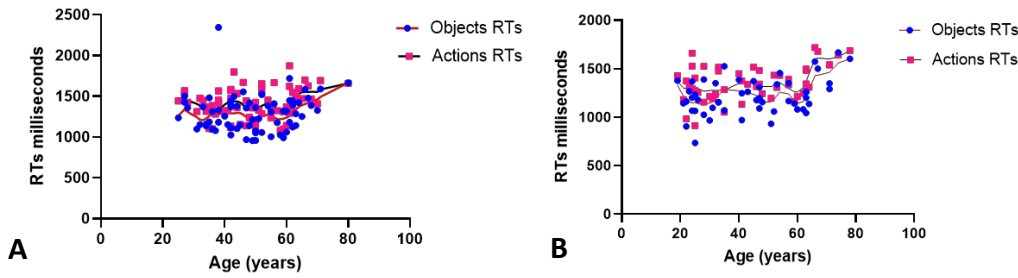


Figure 23: Correlation between age and RTs.

(A) In the FXPCs group no correlation is found between the independent variable age and the RTs for the picture naming task in either object or actions. (B) In the CP group a moderate correlation is present between the independent variable age and the RTs of actions naming in the CP group, indicating that as the age increase so does RTs in naming actions.

We further explore these results by categorising the data per modality. The pattern is no different from the overall analysis as there is no correlation between the FXPCs responses in either of the Picture naming condition (objects or actions) and age, nor for the in-person or online participant, and in the CP group moderate positive correlations were found, that however were not statistically significant. All the other correlations were weak and non-significant as shown in (Figure 24).

Categorisation factor	First variable		Second variable	n.	Correlation Coefficient (r)	p-value
FXPCs in person	Age (years)	vs.	Objects RTs	14	0.011	0.9702
FXPCs in person	Age (years)	vs.	Actions RTs	14	0.126	0.6689
FXPCs online	Age (years)	vs.	Objects RTs	55	0.186	0.1730
FXPCs online	Age (years)	vs.	Actions RTs	54	0.239	0.0817
CP in person	Age (years)	vs.	Objects RTs	17	0.381	0.1313
CP in person	Age (years)	vs.	Actions RTs	17	0.151	0.5624
CP online	Age (years)	vs.	Objects RTs	34	0.142	0.4217
CP online	Age (years)	vs.	Actions RTs	34	0.328	0.0585

Figure 24: Summary of correlations between age and RTs divided by modalities in the picture naming task.

This table illustrates the correlation between age and the RTs of the different conditions in the picture naming task divided by modality of task administration. No significant correlation is identified when modalities are analysed separately.

Picture naming task (accuracy)

We then performed the same kind of analysis for accuracy, and we investigated the errors and disfluencies potential correlations with age:

For FXPCs, only one significant correlation was found: the correlation between age and the percentage of errors for objects was $r(68) = -0.2410$, $p = 0.0445$

indicating a moderate negative correlation that was statistically significant (Figure 25).

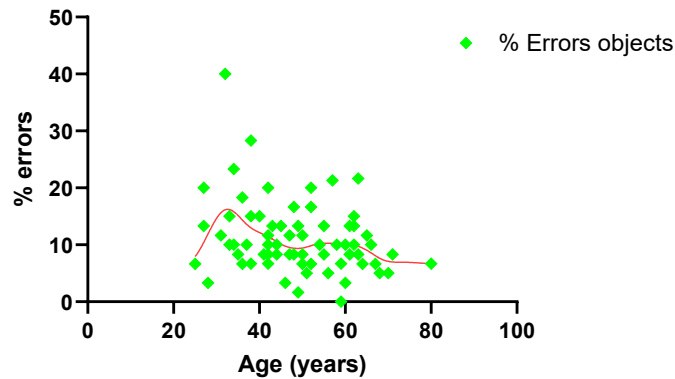


Figure 25: Correlation between age and errors in the objects condition in FXPCs. Age of FXPCs and percentage of errors in the object condition in-person and online combined, were significantly correlated, indicating a potential advantage in accuracy for older individuals in the object condition.

All the other correlations were not significant as shown in Table 13.

Categorisation factor	First variable		Second variable	n	Correlation Coefficient	p-value
FXPCs	Age (years)	vs.	% of errors and disfluencies	70	-0.2124	0.0776
FXPCs	Age (years)	vs.	% of errors and disfluencies actions	70	0.03063	0.8013
FXPCs	Age (years)	vs.	% of dysfluencies objects	70	-0.08154	0.5022
FXPCs	Age (years)	vs.	% of errors actions	70	0.08862	0.4657
CP	Age (years)	vs.	% of dysfluencies actions	70	0.03468	0.7756
CP	Age (years)	vs.	% of errors and disfluencies objects	51	0.07943	0.5796
CP	Age (years)	vs.	% of errors and disfluencies	51	0.1591	0.2648
CP	Age (years)	vs.	% of errors objects	51	-0.04276	0.7657
CP	Age (years)	vs.	% of dysfluencies objects	51	0.1638	0.2507
CP	Age (years)	vs.	% of errors actions	51	0.2705	0.0549
	Age (years)	vs.	% of dysfluencies actions	51	0.1103	0.4408

Table 13: Summary of correlations between age and accuracy.

The table illustrates the correlation between age and accuracy in the different conditions (objects and actions) in the picture naming task. Only the correlation between age and the percentage of errors in the objects condition was significant in the FXPCs group.

The correlation indicates that as age increases the percentage of errors decreases. This might reflect a compensatory mechanism, put in place by older individuals

to balance difficulties in certain cognitive mechanisms. Next, we separated analysis of correlation between age and accuracy per modality of task administration. None of the correlations with age reached statistical significance in the in-person modality as shown in Table 14.

Categorisation factor	First variable		Second variable	n	Correlation Coefficient (ρ)	p-value
FXPCs in person	Age (years)	vs.	% of errors and disfluencies objects	70	-0.3947	0.1619
FXPCs in person	Age (years)	vs.	% of errors and disfluencies actions	70	-0.2365	0.4115
FXPCs in person	Age (years)	vs.	% of errors objects	70	-0.1755	0.5443
FXPCs in person	Age (years)	vs.	% of disfluencies objects	70	-0.4817	0.0826
FXPCs in person	Age (years)	vs.	% of errors actions	70	0.0575	0.8444
FXPCs in person	Age (years)	vs.	% of disfluencies actions	70	-0.0011	0.9985
CP in person	Age (years)	vs.	% of errors and disfluencies objects	51	0.2068	0.4225
CP in person	Age (years)	vs.	% of errors and disfluencies actions	51	0.1087	0.6761
CP in person	Age (years)	vs.	% of errors objects	51	0.2003	0.4374
CP in person	Age (years)	vs.	% of disfluencies objects	51	0.1816	0.4811
CP in person	Age (years)	vs.	% of errors actions	51	0.0062	0.9819
CP in person	Age (years)	vs.	% of disfluencies actions	51	0.2428	0.3442

Table 14: Summary of correlations between age and accuracy in-person.

The table illustrates the correlation between age and accuracy in the different conditions (objects and actions) in the picture naming task online. A moderate negative correlation was found between age and the percentage of errors in the object condition in FXPCs, while a moderate positive correlation was present between age and the percentage of errors in the action condition in CP.

Then we correlated age and accuracy in the online modality: no significant correlations between FXPCs online and accuracy was found as shown in Table 15. One moderate significant correlation was found in the CP group between age and the percentage of errors in the action condition, $r(49) = 0.4013$, $p = 0.0186$ indicating a moderate positive correlation that was statistically significant (Figure 26).

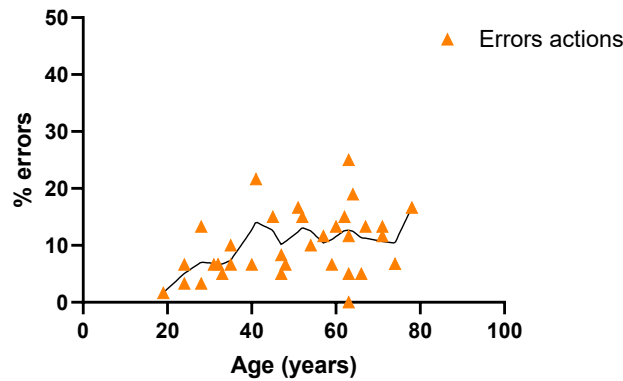


Figure 26: Correlation between age and percentage of errors in the action condition online in CP.

Age was significantly correlated with the percentage of errors in the action condition in the CP group, showing a potential disadvantage for older individual in accuracy in the action condition.

Categorisation factor	First variable		Second variable	n	Correlation Coefficient	p-value
FXPCs online	Age (years)	vs.	% of errors and disfluencies objects	70	-0.1843	0.1740
FXPCs online	Age (years)	vs.	% of errors and disfluencies actions	70	0.1021	0.4541
FXPCs online	Age (years)	vs.	% of errors objects	70	-0.2581	0.0548
FXPCs online	Age (years)	vs.	% of dysfluencies objects	70	-0.03670	0.7883
FXPCs online	Age (years)	vs.	% of errors actions	70	0.08719	0.5228
FXPCs online	Age (years)	vs.	% of dysfluencies actions	70	0.06012	0.6599
CP online	Age (years)	vs.	% of errors and disfluencies objects	51	-0.1387	0.4339
CP online	Age (years)	vs.	% of errors and disfluencies actions	51	-0.007427	0.9667
CP online	Age (years)	vs.	% of errors objects	51	0.09597	0.5893
CP online	Age (years)	vs.	% of dysfluencies objects	51	-0.1667	0.3460
CP online	Age (years)	vs.	% of errors actions	51	0.4013	0.0186
CP online	Age (years)	vs.	% of dysfluencies actions	51	-0.2011	0.2541

Table 15: Summary of correlations between age and accuracy online.

The table illustrates the correlation between age and accuracy in the different conditions (objects and actions) in the picture naming task online. A moderate negative correlation was found between age and the percentage of errors in the object condition in FXPCs, while a moderate positive correlation was present between age and the percentage of errors in the action condition in CP.

Visual Word Paradigm

The correlation between age and the RTs in the VWP indicated a moderate correlation between age and RTs in both conditions—actions and objects—for

the CP group. However, only a correlation with the object condition was observed for the FXPCs group. In the FXPCs group, the correlation between age and RTs was statistically significant for the object condition $r(67) = 0.3580$, $p = 0.0025$, but not for the action condition $r(67) = 0.1506$, $p = 0.2166$. These results suggest that as age increases so does the time to retrieve objects, but not actions. This potentially indicates different mechanisms between conditions. FXPCs (Figure 27). In contrast, the CP group showed a statistically significant relationship between age and RTs for both conditions. The correlation between age and RTs for objects was $r(48) = 0.4874$, $p = 0.0003$, indicating a positive correlation. For actions, the correlation was $r(48) = 0.3144$, $p = 0.0262$, also demonstrating a positive correlation (Figure 27). These results imply that as age increases, RTs tend to increase too in the CP group for both object and action condition; however, the correlation in the object condition is stronger

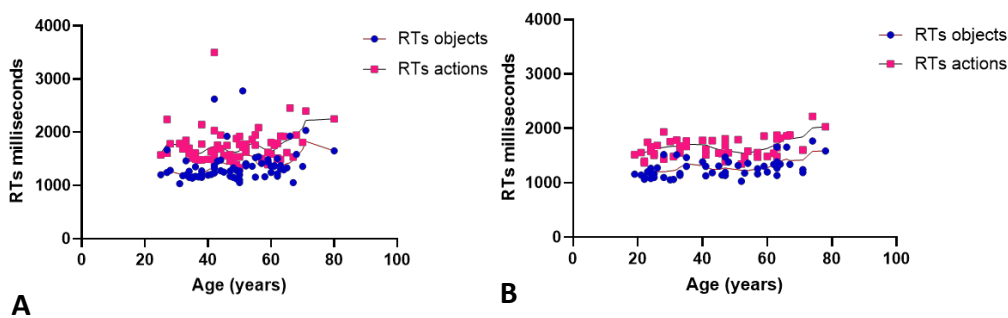


Figure 27: Correlation between age and RTs in the VWP task.

(A) In the FXPCs group the correlation revealed a correlation between RTs and age in the objects condition, indicating that as age increases so does the naming retrieval time. However, no association between age and RTs in the action condition was found. (B) The correlation revealed an association in the CP group between age and RTs in both conditions showing that as age increases RTs become slower.

Subsequent correlation analyses were conducted to examine the groups separately based on testing modalities (in-person vs. online). The results revealed no significant differences between the overall pattern and the results obtained from the in-person and online groups, respectively (Table 16). Overall, the results show that age significantly correlate with RTs in the FXPCs group in the online modality, $r(53) = 0.377$, $p = 0.0045$, in the object condition, suggesting that as age increase so do RTs, while no such relationship is observed for the CP group in either testing modality.

Categorisation factor	First variable		Second variable	n	Correlation Coefficient	p-value
FXPCs in person	Age (years)	vs.	VWP objects	14	0.141	0.6307
FXPCs in person	Age (years)	vs.	VWP actions	14	0.093	0.7531
FXPCs online	Age (years)	vs.	VWP objects	55	0.377	0.0045
FXPCs online	Age (years)	vs.	VWP actions	55	0.190	0.1652
CP in person	Age (years)	vs.	VWP objects	16	0.377	0.1500
CP in person	Age (years)	vs.	VWP actions	16	-0.106	0.6959
CP online	Age (years)	vs.	VWP objects	34	0.336	0.0519
CP online	Age (years)	vs.	VWP actions	34	0.313	0.0720

Table 16: Summary of correlations between age and VWP RTs divided by modality. This table gives an overview of correlation analysis performed between age and RTs in the VWP task divided by modality of task administration. Only a moderate correlation was found in the FXPCs group online with the object indicating that as age increases object RTs also increase.

Picture description

The Spearman rank correlation analysis examining the relationship between age and various disfluencies measures (FLUCALC) across the CP and FXPCs groups yielded significant findings. In the CP group, a positive correlation was observed between age and the whole word repetition (WWR_1) variable, expressed as $r(45) = 0.337$, $p = 0.0207$. This indicates a moderate, statistically significant relationship, suggesting that as age increases, so does the percentage of the disfluency measure WWR_1. Additionally, the correlation between age and mean of repetition unit (MeanRU) revealed $r(45) = 0.321$, $p = 0.0278$, further indicating a moderate positive relationship. These results imply that older individuals in the CP group tend to have a higher percentage of word repetition disfluency measures in the picture description task, highlighting the potential influence of age on linguistic abilities in control participants. Conversely, in the FXPCs group, the analysis revealed a negative correlation between age and the filled pauses (Filledpauses_1: pauses filled by segments like “*uhm, ehm, mmm*” etc) measure, represented as $r(62) = -0.356$, $p = 0.0039$. This finding suggests that as age increases, the frequency of filled pauses decreases, indicating a potential compensatory mechanism that led to improvement in fluency or a more refined control over speech production with age. Additionally, a moderate negative correlation was observed between age and the typical disfluencies (TD_1: such as sum of phrase repetitions, word revisions, phrase revisions, pauses, phonological fragments, and filled pauses) measure, expressed as $r(62)$

=-0.246, $p=0.0501$, that was not significant, but it narrowly missed it. In summary, these correlation analyses reveal distinct patterns across the CP and FXPCs groups, highlighting the complex interplay between age and linguistic performance. In the CP group, age appears to be correlated with an increase in certain types of disfluencies, while in the FXPCs group, increased age is linked to a reduction in disfluencies, suggesting potential improvements in specific aspects of language production as individuals age. However, these correlations were not strong enough to definitive conclusions.

We then ran a series of Spearman rank correlations between the more general language measures (EVAL) and age in the CP and FXPCs group. In the CP group, a moderate positive correlation was found between age and percentage of modal verbs, expressed as $r(45) = 0.312$, $p=0.0330$. This suggests that as age increases, individuals tend to produce a higher percentage of modal verbs in their speech, indicating a potential increase in linguistic complexity with age. In the FXPCs group, a significant positive correlation was observed between age and percentage adverbs, expressed as $r(62) = 0.2620$, $p=0.03660$. This result suggests that as individuals in the FXPCs group age, their speech contains a higher proportion of adverbs, which may reflect changes in the way they structure or enrich their language with age. These findings highlight distinct age-related patterns in linguistic production across the CP and FXPCs groups, indicating that as individuals age, their speech might become more linguistically complex in certain aspects. However, these correlations were moderate to weak, hence any interpretation would need to be confirmed by further investigations.

Correlation with CGG repeat size

Picture naming

The following Spearman Rank correlations examine the relationship between CGG repeats and RTs for objects and actions, both in person and online. For overall conditions (combined in-person and online) one significant correlation was found between CGG repeats and object RTs was $r(41) = 0.4043$, $p=0.00079$ indicating a moderate positive correlation that was statistically significant (Figure 28). While the correlation between CGG repeats and action RTs was $r(40) = 0.2364$, $p=0.1318$ indicating a moderate non-significant positive correlation. In the in-person condition a moderate correlation with objects RTs was found that was not significant, $r(5) = 0.5225$, $p=0.2405$, and

no correlation with action RTs, $r(5) = -0.2523$, $p = 0.5825$. In the online condition a moderate significant correlation was found again with objects RTs, $r(34) = 0.4103$, $p = 0.0129$, while the correlation with actions RTs was weak and not significant $r(33) = 0.2775$, $p = 0.1065$.

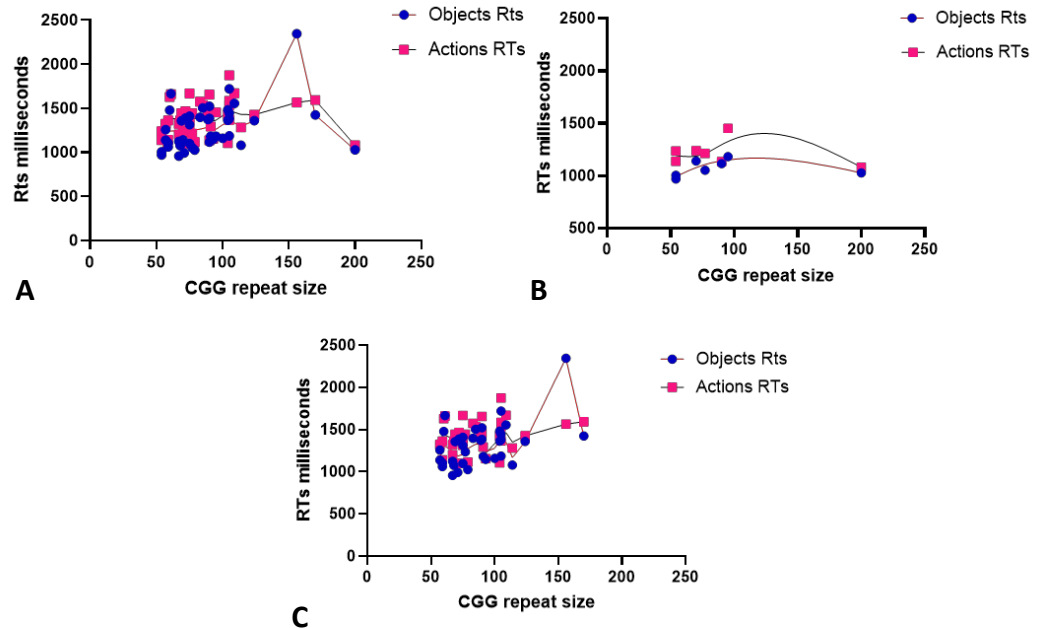


Figure 28: Correlation between CGG repeat size and RTs.

(A) In the in-person and online modalities combined a correlation is present between CGG repeat size and RTs in the object condition where to a higher number of repeats correspond slower RTs. (B) In the in-person modality a correlation is present between CGG repeat size and RTs in the object condition where to a higher number of repeats correspond slower RTs. (C) In the online modality a correlation is present between CGG repeat size and the object condition where to a higher number of repeats correspond slower RTs.

Subsequently, we compared the CGG repeat size with accuracy which did not yield any significant correlation in any modality of task administration as shown in Table 17, indicating potentially no connection between accuracy and CGG repeat size.

Categorisation factor	First variable		Second variable	n	Correlation Coefficient	p-value
Overall	CGG repeats	vs.	Errors and dysfluencies objects	44	0.131	0.3972
Overall	CGG repeats	vs.	Errors and dysfluencies actions	44	0.073	0.6380
Overall	CGG repeats	vs.	Errors objects	44	-0.071	0.6462
Overall	CGG repeats	vs.	Dysfluencies objects	44	0.190	0.2163
Overall	CGG repeats	vs.	Errors actions	44	-0.088	0.5681
Overall	CGG repeats	vs.	Dysfluencies actions	44	0.100	0.5180
In-person	CGG repeats	vs.	Errors and dysfluencies objects	7	0.164	0.7259
In-person	CGG repeats	vs.	Errors and dysfluencies actions	7	0.064	0.8922
In-person	CGG repeats	vs.	Errors objects	7	0.072	0.8780
In-person	CGG repeats	vs.	Dysfluencies objects	7	-0.082	0.8616
In-person	CGG repeats	vs.	Errors actions	7	-0.187	0.6881
In-person	CGG repeats	vs.	Dysfluencies actions	7	-0.037	0.9377
In-person	CGG repeats	vs.	Errors and dysfluencies objects	37	0.164	0.3321
Online	CGG repeats	vs.	Errors and dysfluencies actions	37	0.096	0.5725
Online	CGG repeats	vs.	Errors objects	37	-0.094	0.5798
Online	CGG repeats	vs.	Dysfluencies objects	37	0.253	0.1312
Online	CGG repeats	vs.	Errors actions	37	-0.054	0.7514
Online	CGG repeats	vs.	Dysfluencies actions	37	0.116	0.4933

Table 17: Summary of correlations between CGG repeat size and accuracy in the picture naming task.

The table illustrates the correlation between CGG repeat size and accuracy in the picture naming task in the different modalities. No correlations were found between CGG repeats and accuracy.

Visual Word Paradigm

Subsequently, a correlation analysis was conducted to assess the relationship between CGG repeat length and RTs in the VWP task. The analysis was performed across both object and action conditions and indicated no meaningful correlations with objects RTs $r(41) = 0.113$, $p = 0.4713$, and with actions RTs $r(41) = 0.131$, $p = 0.4015$. These findings suggest that CGG repeat length does not play a significant role in affecting RTs in either condition of the VWP task (Table 18).

Categorisation factor	First variable		Second variable	n	Correlation Coefficient	p-value
Overall	CGG repeats	vs.	VWP objects	43	0.113	0.4713
Overall	CGG repeats	vs.	VWP actions	43	0.131	0.4015

Table 18: Correlation between CGG repeat size and VWP RTs in-person and online combined.

This tables illustrates the correlation analysis performed between CGG repeat size and RTs in the VWP task for both conditions (objects and actions), combining in-person and online modality. No significant correlations are identified here, potentially indicating that CGG repeat does not play a role in RTs in VWP.

A subsequent series of correlations were performed separating between in-person and online modalities and no correlations were identified in-person or online as shown in Table 19. Overall, these findings indicate that CGG repeat length does not consistently correlate with RTs across both the in-person and online modalities, and across objects and actions conditions in the VWP task.

Categorisation factor	First variable		Second variable	n	Correlation Coefficient	p-value
In person	CGG	vs.	VWP objects	7	0.252	0.5852
In person	CGG	vs.	VWP actions	7	-0.342	0.4523
online	CGG	vs.	VWP objects	36	0.110	0.5215
online	CGG	vs.	VWP actions	36	0.275	0.1048

Table 19: Summary of correlation between CGG repeat size and VWP RTs divided by modality.

No significant correlation between CGG repeat size and VWP is identified when divided by modality.

Picture description

We performed a correlation analysis between all EVAL and FLUCAL measures and the number of CGG repeats in the FXPCs group. No correlations with the EVAL general language measures were found. Conversely, in the FLUCAL measures of dysfluencies we found moderate significant positive correlations between CGG repeats and pauses, filled pauses and typical dysfluencies (TD). Specifically, the correlation was $r(41) = 0.363$, $p = 0.0169$ for pauses, $r(41) = 0.385$, $p = 0.0109$ for filled pauses, and $r(41) = 0.383$, $p = 0.0112$ for TD, indicating that as the number of CGG repeats increases, the occurrence of certain types of dysfluencies also tends to increase. We also found moderate negative correlations between CGG repeat size and the syllables per minute (syllablesmin) measure $r(41) = -0.418$, $p = 0.0053$, and the words per minute measure (wordsmin) $r(41) = -0.325$, $p = 0.0332$, indicating that as the CGG repeat size increases the syllables and words per minute decrease potentially

indicating a more dysfluent speech or difficulties in articulation. This suggests that higher CGG repeat expansions may be associated with increased speech disfluency in the FXPCs group.

Correlation with other demographic features

A series of Spearman rank correlation analyses were performed to examine the relationships between various demographic features (gender, medical history, medications, and languages spoken) and their performance on RTs in picture naming and visual world paradigm (VWP) tasks. Here are the key findings: Gender was significantly correlated with multiple task measures: a positive correlation between gender and objects RTs in picture naming ($r(67) = 0.2830$, $p = 0.0184$) shows that males participants had longer RTs compared to females in naming objects, similarly, a positive correlation was found between gender and actions RTs in picture naming ($r(66) = 0.2576$, $p = 0.0340$), indicating longer RTs for males in action-naming tasks as well. Gender also correlated positively with VWP objects ($r(67) = 0.3864$, $p = 0.0010$) and VWP actions ($r(67) = 0.2568$, $p = 0.0331$), meaning that males tended to spend more time processing both objects and actions in the visual world paradigm. These findings suggest that gender differences play a role in how participants engage with and respond to these linguistic tasks, with males showing generally longer RTs.

Medical history, and medications did not show any significant associations with the linguistic tasks, potentially indicating that these aspects do not significantly affect RTs in these tasks. The number of languages spoken revealed significant negative correlations: a negative correlation between languages spoken and objects RTs in picture naming ($r(67) = -0.2391$, $p = 0.0478$) suggests that participants who speak more languages performed faster in object-naming tasks. A stronger negative correlation between languages spoken and actions RTs in picture naming ($r(66) = -0.3059$, $p = 0.0112$) further indicates that multilingual participants also showed faster RTs when naming actions (Figure 29). However, there were no significant correlations between languages spoken and VWP objects ($r(67) = -0.1359$, $p = 0.2657$) or VWP actions ($r(67) = -0.0869$, $p = 0.4778$). These findings might imply that multilingualism may contribute to faster cognitive and linguistic processing, particularly in tasks requiring the naming of objects and actions.

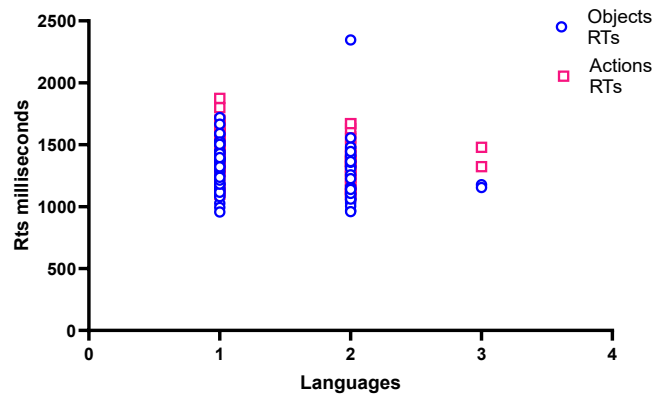


Figure 29: Correlation of RTs and languages in the FXPCs group.

This figure shows the presence of a negative correlation between RTs in the picture naming and languages spoken where those who speak more languages seems to have faster RTs. Each number in the Languages corresponds to a category: 1= only English, 2=English and other languages, 3= non-native speakers, fluent in English.

Subsequently we run similar Spearman rank correlations for the CP group to explore the relationships between the participant demographic features (gender, medical history, medications, and languages spoken) and their performance on RTs in picture naming and visual world paradigm tasks. Gender and medical history in this case were not significantly correlated with any of the measures as shown in Table 20. Medications were moderately correlated with some measures: a moderate significant correlation was observed between medications and VWP objects ($r(67) = 0.4499$, $p = 0.0010$), suggesting that medication use may correlate with object processing in the VWP task. A similar positive correlation was found between medications and VWP actions ($r(67) = 0.3966$, $p = 0.0044$), indicating a correlation with the use of certain medication and action processing (Figure 30). No significant correlations were found for medications and objects RTs in picture naming ($r(67) = 0.2146$, $p = 0.1304$) or actions RTs in picture naming ($r(66) = 0.1198$, $p = 0.4025$).

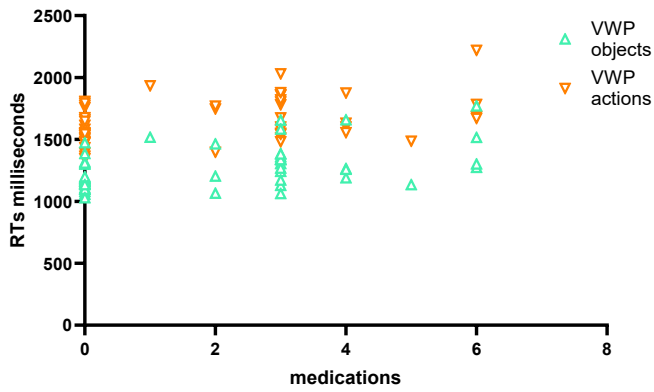


Figure 30: Correlation between medications and VWP in the CP group.

This figure shows a moderate correlation between RTs and medications and particularly high RTs seems to correspond to psychoactive and steroids intake. Each number corresponds to a category: none 0, steroids 1, psychoactive 2, other 3, steroids and other 4, psychoactive and steroids 5, psychoactive and others 6, psychoactive steroids others 7.

The number of languages spoken also revealed notable results: a significant negative correlation between languages spoken and VWP objects ($r(67) = -0.5032$, $p = 0.0002$) indicates that multilingual participants processed objects more quickly (Figure 31). Similarly, languages spoken negatively correlated with VWP actions ($r(67) = -0.4437$, $p = 0.0012$), suggesting faster action processing in multilingual participants (Figure 31). No significant correlations were found for languages spoken and objects RTs in picture naming ($r(67) = -0.1751$, $p = 0.2189$) or actions RTs in picture naming ($r(66) = 0.0367$, $p = 0.7981$).

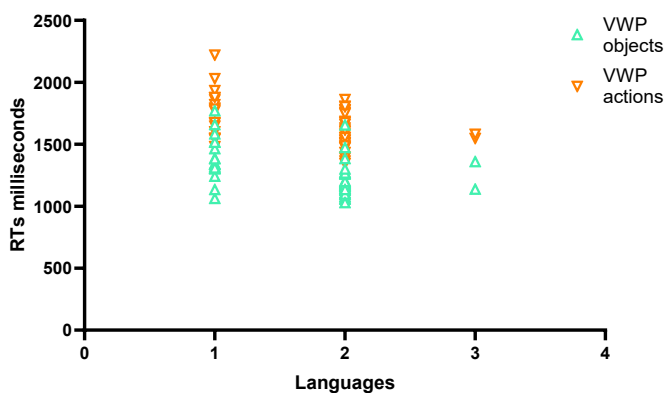


Figure 31: Correlation between languages and VWP in the CP group.

This figure shows negative correlations between languages and RTs in the CP group. Participants speaking more than one language seems to have faster RTs.

Categorisation factor	First variable		Second variable	n	Correlation Coefficient	p-value
CP	gender	vs.	Objects RTs Picture naming	69	-0.01422	0.9219
CP	gender	vs.	Actions RTs Picture naming	68	0.02293	0.8731
CP	gender	vs.	VWP objects	69	-0.01422	0.9219
CP	gender	vs.	VWP actions	69	0.02686	0.8531
CP	Medical history	vs.	Objects RTs Picture naming	69	0.08168	0.5688
CP	Medical history	vs.	Actions RTs Picture naming	68	-0.1387	0.3319
CP	Medical history	vs.	VWP objects	69	0.1741	0.2266
CP	Medical history	vs.	VWP actions	69	0.2524	0.0770
CP	Medications	vs.	Objects RTs Picture naming	69	0.2146	0.1304
CP	Medications	vs.	Actions RTs Picture naming	68	0.1198	0.4025
CP	Medications	vs.	VWP objects	69	0.4499	0.0010
CP	Medications	vs.	VWP actions	69	0.3966	0.0044
CP	Languages	vs.	Objects RTs Picture naming	69	-0.1751	0.2189
CP	Languages	vs.	Actions RTs Picture naming	68	0.03671	0.7981
CP	Languages	vs.	VWP objects	69	-0.5032	0.0002
CP	Languages	vs.	VWP actions	69	-0.4437	0.0012

Table 20: Correlation analysis of demographic factors and RTs in the CP group.

This table reports results of a series of Spearman Rank correlations performed between picture naming and VWP RTs in the CP group and participants' demographic features such as gender, medical history, medications used, and languages spoken.

Subsequently we ran a series of Spearman Rank correlations to identify the correlations between demographic features and accuracy (percentage of errors and percentage of disfluencies) in the picture naming task. Gender revealed some moderate correlations: a significant negative correlation was found between gender and errors/disfluencies in object naming ($r(68) = -0.267$, $p = 0.0253$). This suggests that males tended to produce fewer errors and disfluencies compared to females when naming objects. Conversely, a significant positive correlation was found between gender and errors in action naming ($r(68) = 0.261$, $p = 0.0292$). This indicates that males made more errors when naming actions compared to females (Figure 32).

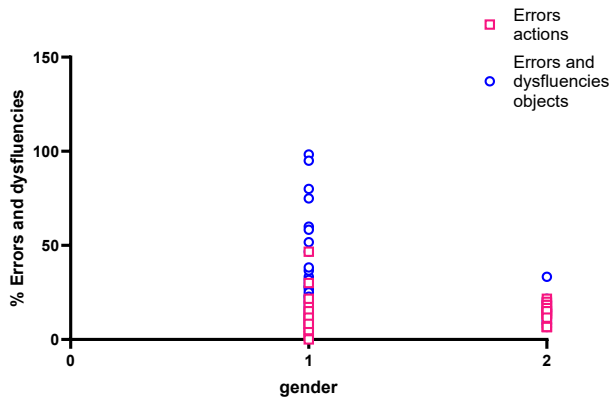


Figure 32: Correlation between accuracy and gender in the FXPCs group.

This figure shows a moderate negative correlation between gender and accuracy in the FXPCs group in objects, where males (2) seem to produce fewer errors than females. While in the action condition the opposite pattern is present, where males made more errors than females (1): category 1= F and 2=M.

Medical history, and medications did not reveal any significant correlations, potentially indicating no influence of these features in the linguistic results. Languages showed a significant correlation with the errors in action naming ($r(68) = 0.385$, $p = 0.0053$), indicating that individuals who spoke more languages tended to make more errors during action naming (Figure 33).

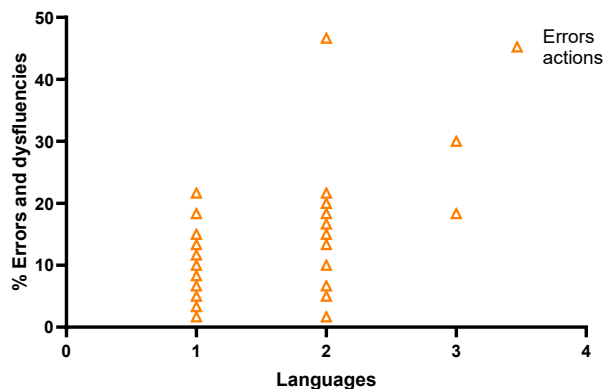


Figure 33: Correlation between accuracy and languages in the FXPCs group.

This figure shows a moderate correlation between languages and accuracy in the FXPCs group, potentially indicating that multilingualism yielded more errors in the action condition.

All the other correlations were not significant as shown in Table 21.

Categorisation factor	First variable		Second variable	n	Correlation Coefficient	p-value
FXPCs	gender	vs.	Errors and dysfluencies objects	70	-0.2673	0.0253
FXPCs	gender	vs.	Errors and dysfluencies actions	70	-0.04831	0.6913
FXPCs	gender	vs.	Errors objects	70	-0.02429	0.8418
FXPCs	gender	vs.	Dysfluencies objects	70	-0.2145	0.0745
FXPCs	gender	vs.	Errors actions	70	0.2608	0.0292
FXPCs	gender	vs.	Dysfluencies actions	70	-0.1183	0.3294
FXPCs	Medical history	vs.	Errors and dysfluencies objects	70	-0.07613	0.5954
FXPCs	Medical history	vs.	Errors and dysfluencies actions	70	-0.007630	0.9576
FXPCs	Medical history	vs.	Errors objects	70	-0.2383	0.0922
FXPCs	Medical history	vs.	Dysfluencies objects	70	-0.04874	0.7341
FXPCs	Medical history	vs.	Errors actions	70	-0.09808	0.4935
FXPCs	Medical history	vs.	Dysfluencies actions	70	-0.03338	0.8161
FXPCs	Medications	vs.	Errors and dysfluencies objects	70	-0.04555	0.7510
FXPCs	Medications	vs.	Errors and dysfluencies actions	70	-0.05036	0.7256
FXPCs	Medications	vs.	Errors objects	70	-0.1958	0.1685
FXPCs	Medications	vs.	Dysfluencies objects	70	0.1216	0.3955
FXPCs	Medications	vs.	Errors actions	70	-0.09776	0.4949
FXPCs	Medications	vs.	Dysfluencies actions	70	0.04283	0.7654
FXPCs	Languages	vs.	Errors and dysfluencies objects	70	0.2206	0.1198
FXPCs	Languages	vs.	Errors and dysfluencies actions	70	0.07185	0.6163
FXPCs	Languages	vs.	Errors objects	70	0.1184	0.4081
FXPCs	Languages	vs.	Dysfluencies objects	70	0.08663	0.5455
FXPCs	Languages	vs.	Errors actions	70	0.3846	0.0053
FXPCs	Languages	vs.	Dysfluencies actions	70	-0.2437	0.0848

Table 21: Correlation between accuracy and demographic factors in the FXPCs group.

This table reports results of a series of Spearman Rank correlations performed between picture naming accuracy in the FXPCs group and participants' demographic features such as gender, medical history, medications used, and languages spoken.

Lastly, we explored the correlation between the demographic factors against errors and disfluencies in object and action naming in the CP group, which however did not reveal any significant correlation as shown in Table 22. Only the correlation between medication uses and errors in object naming was $r(49) = 0.269$, $p = 0.0560$, indicating a weak but non-significant relationship.

Categorisation factor	First variable		Second variable	n	Correlation Coefficient	p-value
CP	gender	vs.	Errors and dysfluencies	51	0.03876	0.7871
CP	gender	vs.	Errors and dysfluencies	51	0.06005	0.6755
CP	gender	vs.	Errors objects	51	0.01363	0.9244
CP	gender	vs.	Dysfluencies objects	51	0.03701	0.7965
CP	gender	vs.	Errors actions	51	0.1886	0.1851
CP	gender	vs.	Dysfluencies actions	51	0.06834	0.6337
CP	Medical history	vs.	Errors and dysfluencies	51	-0.1189	0.4060
CP	Medical history	vs.	Errors and dysfluencies	51	-0.04075	0.7765
CP	Medical history	vs.	Errors objects	51	-0.1669	0.2419
CP	Medical history	vs.	Dysfluencies objects	51	-0.03188	0.8243
CP	Medical history	vs.	Errors actions	51	-0.004065	0.9774
CP	Medical history	vs.	Dysfluencies actions	51	-0.06740	0.6384
CP	Medications	vs.	Errors and dysfluencies	51	0.2000	0.1593
CP	Medications	vs.	Errors and dysfluencies	51	0.02051	0.8864
CP	Medications	vs.	Errors objects	51	0.2693	0.0560
CP	Medications	vs.	Dysfluencies objects	51	-0.02309	0.8722
CP	Medications	vs.	Errors actions	51	0.1624	0.2550
CP	Medications	vs.	Dysfluencies actions	51	-0.03472	0.8089
CP	Languages	vs.	Errors and dysfluencies	51	0.01104	0.9387
CP	Languages	vs.	Errors and dysfluencies	51	-0.1736	0.2232
CP	Languages	vs.	Errors objects	51	0.1550	0.2774
CP	Languages	vs.	Dysfluencies objects	51	-0.06902	0.6303
CP	Languages	vs.	Errors actions	51	0.1362	0.3407
CP	Languages	vs.	Dysfluencies actions	51	-0.2191	0.1224

Table 22: Correlation between accuracy and demographic factors in the CP group. This table reports results of a series of Spearman Rank correlations performed between picture naming accuracy in the CP group and participants' demographic features such as gender, medical history, medications used, and languages spoken.

Correlation between latency and accuracy per word.

Lastly, we performed a series of Spearman Rank correlations for the picture naming task to correlate accuracy and latency per word, to verify whether the inherent features of the word were correlated with accuracy and latency in the different groups. To perform this analysis, we computed the average RTs for each word per group, and the percentage of errors was the same one calculated in the qualitative analysis, using as denominator the total number of errors. For FXPCs in the objects condition, the correlation between the average RTs per trial (word) and the percentage of errors per trial was $r(58) = 0.8501$, $p < 0.0001$ (Figure 34). This indicates a strong positive correlation, which is statistically significant. This strong correlation is confirmed even when analysis was performed separating by modality of task administration, in-person $r(58) = 0.8200$, $p < 0.0001$ and online $r(58) = 0.8416$, $p < 0.0001$. The same pattern is present in the CP group, with both modalities combined $r(58) = 0.863$, $p < 0.0001$, confirmed by the in-person $r(58) = 0.754$, $p < 0.0001$ and online modalities $r(58) = 0.831$, $p < 0.0001$. This suggests that as the average RTs for words increase the percentage of errors in words also increase, meaning that the words with the highest number of errors were also the words which took more time to name overall.

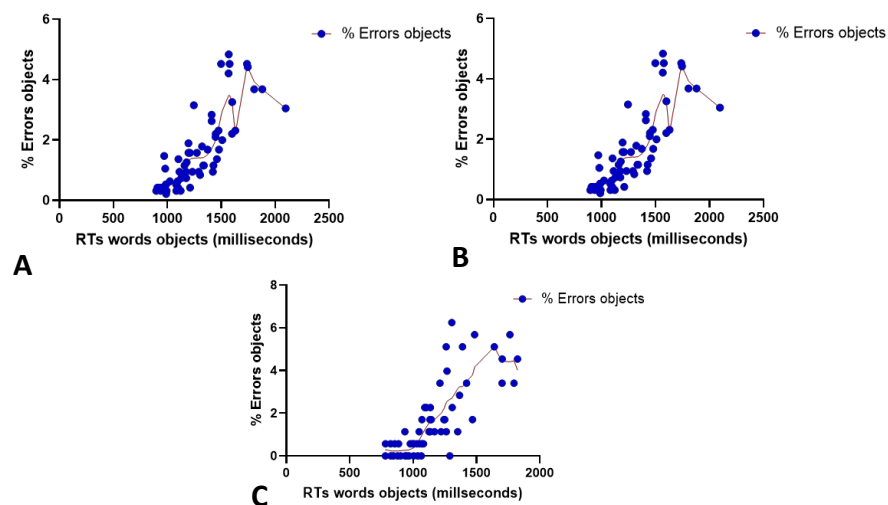


Figure 34: Correlation between latency and accuracy per word in the objects condition in FXPCs.

The strong and positive correlation is present in the in-person and online modalities combined (A), in the in-person modality (B) and in the online modality (C). It indicates that slower RTs per word correspond to a higher percentage of errors per word, potentially meaning that the most difficult words require more time to be named and generate more errors.

Similar correlations were also found in the action condition. The correlation was significant for FXPCs with both modalities combined, $r(58) = 0.898$, $p < 0.0001$, in the in-person modality $r(58) = 0.786$, $p < 0.0001$, and in the online modality $r(58) = 0.901$, $p < 0.0001$. In the CP group similar results were found in both modalities combined $r(58) = 0.908$, $p < 0.0001$, in-person $r(58) = 0.824$, $p < 0.0001$ and online $r(58) = 0.884$, $p < 0.0001$. This again suggests that as the average RTs per trial increase the percentage of errors per trial also increases, as shown in the object condition. A similar pattern is present in the actions as well as in the objects condition, showing that in general the words with a highest percentage of errors were also the ones with slower RTs.

Correlation with psycholinguistic features

Picture naming

Our final correlation was done to investigate potential correlations between the main psycholinguistics features used to match the words between objects and actions, known to influence lexical access, and latency and accuracy^{xxvii}. In the object condition, several significant correlations were observed between average RTs and various psycholinguistic variables in both modalities of task administration combined. The strongest correlation was a negative one present with name agreement (elex1) in the FXPCs group $r(58) = -0.627$, $p < 0.0001$, and in the CP group $r(58) = -0.651$, $p < 0.0001$, indicating that as name agreement increases RTs decreases, suggesting that higher agreement leads to quicker responses. Moderate negative correlations were also found with object complexity (ovcjpg), in the FXPCs group $r(58) = -0.265$, $p = 0.0406$, and in the CP group $r(58) = -0.292$, $p = 0.0237$, and in word frequency (Inefreq), in the FXPCs group $r(58) = -0.371$, $p = 0.0035$, and in the CP group $r(58) = -0.306$, $p = 0.0174$ suggesting that participants responded faster as word frequency increased and as object complexity increased. A moderate positive correlation was found with age of acquisition (ecdi), in the FXPCs group $r(58) = 0.430$,

^{xxvii} The psycholinguistic features are defined in 0. Elex1: Percent name agreement as defined by the IPNP database, represents the proportion of all valid trials with a codable response. Inefreq: frequency counts are taken from the CELEX lexical database. Ovcjpg: estimates of object visual complexity were obtained by the picture itself converting the picture into various graphical files with an Image software and then complexity was analysed. Ecdi: Objective AoA (Age of Acquisition) the IPNP database established the different AOA from published norms of the American version of the MacArthur Communicative Development Inventories.
<https://crl.ucsd.edu/experiments/ipnp/dataquery/queryfull.php>

$p=0.0006$, and in the CP group $r(58) = 0.412$, $p = 0.0011$, indicating that words acquired at an earlier age are associated with faster RTs, reflecting a possible effect of word familiarity. Concerning accuracy, once again name agreement was strongly correlated in the FXPCs group $r(58) = -0.684$, $p < 0.0001$, and in the CP group $r(58) = -0.737$, $p < 0.0001$, suggesting that higher name agreement is associated with a lower percentage of errors, highlighting the importance of agreement in reducing errors during processing. Negative correlations were not found with object complexity in the FXPCs group $r(58) = -0.353$, $p=0.0057$, and in the CP group $r(58) = -0.322$, $p = 0.0121$, potentially indicating fewer errors for more complex objects. A moderate positive correlation was found with age of acquisition in the FXPCs group $r(58) = 0.335$, $p=0.0088$, and in the CP group $r(58) = 0.334$, $p = 0.0091$. No correlations between word frequency and accuracy were found in the FXPCs group $r(58) = -0.166$, $p=0.2049$, and in the CP group ($r(58) = -0.180$, $p = 0.1688$, indicating potentially no influence of word frequency in accuracy level. These results show the presence of a correlation between psycholinguistic factors and age of acquisition in both RTs and error rates. Particularly, name agreement shows the strongest correlations with both RTs and errors. The negative correlation with object complexity potentially indicates that higher visual complexity of the picture leads to a better performance, however correlations with this variable were less strong. Similar results were found for both latency and accuracy, except for word frequency, and no differences were found between CP and FXPCs, indicating similar mechanisms in factors influencing picture naming.

When analysing the data separately for online and in-person groups, a similar pattern emerged. Name agreement was again negatively strongly correlated with RTs in the in-person modality in the FXPCs group $r(58) = -0.641$, $p < 0.0001$, and in the CP group, $r(58) = -0.665$, $p < 0.0001$, and in the online modality in the FXPCs group $r(58) = -0.630$, $p < 0.0001$, and in the CP group $r(58) = -0.611$, $p < 0.0001$ suggesting a strong influence of this factor in reducing RTs across groups and modality of task administration. Object complexity was also negatively correlated with RTs in the in-person modality in the FXPCs group, $r(58) = -0.376$, $p = 0.0031$, but not in the CP group where the correlation was not significant $r(58) = -0.201$, $p = 0.1238$, and in the online modality in the FXPCs group $r(58) = -0.284$, $p = 0.0278$, and in the CP group $r(58) = -0.308$, $p = 0.0168$. Word frequency was again negatively correlated with RTs in the in-person

modality in the FXPCs group $r(58) = -0.291$, $p = 0.0240$, and in the CP group $r(58) = -0.292$, $p = 0.0234$, and in the online modality in the FXPCs group $r(58) = -0.342$, $p = 0.0074$, and in the CP group $r(58) = -0.286$, $p = 0.0266$, highlighting a potential influence of word frequency across groups and task administration modality. The positive correlation with age of acquisition was once again confirmed across modalities, in-person in the FXPCs group $r(58) = 0.427$, $p = 0.0007$, and in the CP group $r(58) = 0.397$, $p = 0.0017$, and online in the FXPCs group $r(58) = 0.407$, $p = 0.0012$, and in the CP group $r(58) = 0.411$, $p = 0.0011$. Concerning accuracy the strong negative correlation with name agreement was confirmed in-person in the FXPCs group $r(58) = -0.641$, $p < 0.0001$, and in the CP group $r(58) = -0.694$, $p < 0.0001$, and online in the FXPCs group $r(58) = -0.666$, $p < 0.0001$, and in the CP group $r(58) = -0.675$, $p < 0.0001$. Similarly, the other correlations were also confirmed with Object complexity in-person in the FXPCs group $r(58) = -0.376$, $p = 0.0031$, but not in the CP group $r(58) = -0.200$, $p = 0.1245$, and online in the FXPCs group $r(58) = -0.325$, $p = 0.0113$, and in the CP group $r(58) = -0.344$, $p = 0.0071$. With Word frequency in-person in the FXPCs group $r(58) = -0.291$, $p = 0.0240$, but not in the CP group $r(58) = -0.136$, $p = 0.2993$, but not online in either the FXPCs group $r(58) = -0.126$, $p = 0.3362$, or the CP group $r(58) = -0.157$, $p = 0.2321$. Finally, the positive correlation with age of acquisition was confirmed in-person in the FXPCs group $r(58) = 0.427$, $p = 0.0007$, and in the CP group $r(58) = 0.269$, $p = 0.0375$, and online in the FXPCs group $r(58) = 0.298$, $p = 0.0209$, and in the CP group $r(58) = 0.314$, $p = 0.0145$.

In the action condition name agreement (elex1) consistently showed a moderate to strong, significant negative correlation with RTs in the FXPCs group $r(58) = -0.583$, $P < 0.0001$, and in the CP group $r(58) = -0.589$, $p < 0.0001$, indicating that as name agreement increases, RTs decrease considerably. In contrast Object complexity did not show significant correlations in the FXPCs group $r(58) = 0.125$, $P = 0.3400$, and in the CP group $r(58) = 0.112$, $p = 0.3941$, as well as word frequency in the FXPCs group $r(58) = -0.060$, $P = 0.6478$, and in the CP group $r(58) = -0.095$, $p = 0.4724$, and age of acquisition in the FXPCs group $r(58) = 0.148$, $P = 0.2590$, and in the CP group $r(58) = 0.182$, $p = 0.1648$. Concerning accuracy name agreement still shows strong negative correlations in the FXPCs group $r(58) = -0.567$, $P < 0.0001$, and in the CP group $r(58) = -0.589$, $p < 0.0001$. The other variable instead did not show any significant

correlation, such as object complexity in the FXPCs group $r(58) = 0.226$, $P = 0.0821$, and in the CP group, $r(58) = 0.172$, $p = 0.1901$, word frequency in the FXPCs group $r(58) = -0.041$, $P = 0.7533$, and in the CP group $r(58) = -0.157$, $p = 0.2321$, and age of acquisition in the FXPCs group $r(58) = 0.117$, $P = 0.3713$. An exception is present in age of acquisition in the CP group $r(58) = 0.264$, $p = 0.0418$, however the correlation is not very strong.

The results indicate that most correlations between actions and psycholinguistic variables both in latency and accuracy were weak and non-significant. Notably, a significant negative relationship between both RTs and the percentage of errors with name agreement suggests that this variable might be the only one to play an important role in performance in FXPCs and in CP in action. The other correlations were observed between either RTs or error percentages with the other linguistic variables (ovcjpg, lnefreq, ecdi) were either non-significant or weak.

When separating analysis by modality of task administration we found similar results. Name agreement was again negatively correlated with RTs in-person in the FXPCs group $r(58) = -0.565$, $p < 0.0001$, and in the CP group $r(58) = -0.582$, $p < 0.0001$, and online in the FXPCs group $r(58) = -0.582$, $p < 0.0001$, and in the CP group $r(58) = -0.579$, $p < 0.0001$. All the other variables were not correlated with RTs such as age of acquisition (in-person in the FXPCs group $r(58) = 0.178$, $p = 0.1733$, and in the CP group $r(58) = 0.232$, $p = 0.0743$, and online in the FXPCs group $r(58) = 0.143$, $p = 0.2752$, and in the CP group $r(58) = 0.162$, $p = 0.2162$), object complexity (in-person in the FXPCs group $r(58) = -0.129$, $p = 0.3255$, and in the CP group $r(58) = 0.084$, $p = 0.5251$, and online in the FXPCs group $r(58) = 0.133$, $p = 0.3126$, and in the CP group $r(58) = 0.118$, $p = 0.3677$), word frequency (in-person in the FXPCs group $r(58) = -0.112$, $p = 0.3943$, and in the CP group $r(58) = -0.149$, $p = 0.2563$, and online in the FXPCs group $r(58) = -0.051$, $p = 0.6992$, and in the CP group $r(58) = -0.080$, $p = 0.5430$). Concerning accuracy, a similar pattern is present where only name agreement revealed significant correlations in-person in the FXPCs group $r(58) = -0.533$, $p < 0.0001$, and in the CP group $r(58) = -0.464$, $p = 0.0002$, and online in the FXPCs group $r(58) = -0.572$, $p < 0.0001$, and in the CP group $r(58) = -0.584$, $p < 0.0001$. No other correlation was present with object complexity (in-person in the FXPCs group $r(58) = 0.183$, $p = 0.1611$, and in the CP group $r(58)$

= 0.046, $p = 0.7287$, online in the FXPCs group $r(58) = 0.226$, $p = 0.0823$, and in the CP group $r(58) = 0.213$, $p = 0.1018$), word frequency (in-person in the FXPCs group $r(58) = -0.084$, $p = 0.5255$, and in the CP group $r(58) = -0.130$, $p = 0.3221$, and online in the FXPCs group $r(58) = -0.024$, $p = 0.8541$, and in the CP group $r(58) = -0.125$, $p = 0.3411$) or age of acquisition (in-person in the FXPCs group $r(58) = 0.102$, $p = 0.4371$, and in the CP group $r(58) = 0.253$, $p = 0.0507$, and online in the FXPCs group $r(58) = 0.102$, $p = 0.4371$, and in the CP group $r(58) = 0.233$, $p = 0.0734$). These findings are in line with the results combining in-person and online modality, indicating that name agreement is potentially the only variable correlating with both latency and accuracy.

Visual Word Paradigm

We conducted similar correlation analysis to explore the relationship between RTs and the features of target words in the visual word paradigm. The analysis involved correlating the RTs with the psycholinguistic variables of the target words, such as name agreement (elex1), word frequency (lnfreq), and picture complexity (ovcjpg). We did not consider age of acquisition here as all the target words selected belonged to the same age of acquisition group number 1, which consists of all words acquired within the first year of life, as shown in the IPNP database. In the object condition, in the analysis of both modalities combined we only found a negative correlation with word frequency which did not reach statistical significance in the FXPCs group $r(20) = -0.402$, $p = 0.0635$, but was significant in the CP group $r(20) = -0.476$, $p = 0.0252$. The correlation with name agreement was not significant in the FXPCs group ($r(20) = -0.057$, $p = 0.7998$), or in the CP group $r(20) = -0.068$, $p = 0.7642$, and no correlation was found with object complexity in the FXPCs group $r(20) = 0.022$, $p = 0.9225$ or in the CP group $r(20) = 0.151$, $p = 0.5030$. When we analysed the two modalities separated we found a similar pattern where no correlations were found in-person with name agreement in the FXPCs group $r(20) = 0.062$, $p = 0.7851$, or in the CP group $r(20) = -0.086$, and online in the FXPCs group $r(20) = 0.094$, $p = 0.6781$, and in the CP group $r(20) = -0.027$, $p = 0.9046$. Negative correlations with word frequency were stronger, but it was not significant in-person in the FXPCs group $r(20) = -0.287$, $p = 0.1953$, while it was significant in the CP group ($r(20) = -0.490$, $p = 0.0205$), and online in the FXPCs group $r(20) = -0.416$, $p = 0.0543$ where it was stronger, but still not significant, and in the CP group $r(20) = -0.414$, $p = 0.0554$. No correlation with object complexity was found in-person in the FXPCs group $r(20) =$

0.102, $p = 0.6509$, and in the CP group $r(20) = 0.128$, $p = 0.5697$, and online in the FXPCs group $r(20) = 0.090$, $p = 0.6911$, and in the CP group $r(20) = 0.159$, $p = 0.4806$.

Lastly, we conducted similar correlation analyses for the action condition. In this case, we also included the age of acquisition as variable in our correlations due to the presence of three of the target words belonging to different groups of the ages of acquisition variable (ECDI), as we were unable to identify a perfect match within the database for all the target words in the action condition. The analysis confirmed name agreement as the only variable correlated with results in the analysis of modalities combined in the FXPCs group $r(21) = -0.549$, $p = 0.0066$, and in the CP group $r(21) = -0.496$, $p = 0.0162$, and in the analysis separated by modalities, in-person in the FXPCs group $r(21) = -0.493$, $p = 0.0170$, and in the CP group $r(21) = -0.546$, $p = 0.0070$, and online in the FXPCs group $r(21) = -0.553$, $p = 0.0062$, and in the CP group $r(21) = -0.446$, $p = 0.0328$. None of the other correlations was significant, for word frequency (combined modalities in the FXPCs group $r(21) = 0.078$, $p = 0.7232$ and in the CP group $r(21) = 0.026$, $p = 0.9056$, in-person in the FXPCs group $r(21) = 0.080$, $p = 0.7165$ and in the CP group $r(21) = 0.026$, $p = 0.9056$, and online in the FXPCs group $r(21) = 0.020$, $p = 0.9287$, and in the CP group $r(21) = -0.057$, $p = 0.7967$, and online in the FXPCs group $r(21) = 0.020$, $p = 0.9287$, and in the CP group $r(21) = 0.045$, $p = 0.8385$), age of acquisition (in-person in the FXPCs group $r(21) = 0.005$, $p = 0.9817$, and in the CP group $r(21) = 0.066$, $p = 0.7653$, online in the FXPCs group $r(21) = 0.223$, $p = 0.3066$ and in the CP group $r(21) = 0.203$, $p = 0.3538$), and object complexity (in-person in the FXPCs group $r(21) = 0.256$, $p = 0.2385$, and in the CP group $r(21) = 0.174$, $p = 0.4274$, and online in the FXPCs group $r(21) = 0.129$, $p = 0.5561$, and in the CP group $r(21) = 0.182$, $p = 0.4064$).

Summary of exploratory correlations correlation results between age and linguistic tasks.

Age

In the picture naming task, age showed no correlations with RTs and errors in FXPCs, however one correlation was found in the CP group with actions RTs, while in accuracy age correlated negatively with FXPCs in objects and in the online modality with CP in actions errors. In the visual word paradigm, age moderately correlated with object RTs in FXPCs and both object and action RTs in CP, and no major different patterns were found in the different modalities. In

the picture description task, age correlations varied: the CP group showed increases in disfluencies while the FXPCs group demonstrated reductions in disfluencies like filled pauses and typical dysfluencies. While these correlations were moderate to weak, they highlight distinct age-related linguistic trends across groups. In the CP group age seems to be correlated with increase in certain types of disfluencies, while in the FXPCs group age seems to be related to a reduction in certain types of dysfluencies, suggesting potential compensatory mechanisms. However, most of these correlations were not strong enough to draw definitive conclusions.

CGG repeats

In the picture naming task, in-person group correlations between CGG repeats and RTs were weak and non-significant, while the online group and combined modality showed moderate and significant correlations with for object RTs, but not for action RTs, indicating that higher CGG repeats corresponded to slower RTs in objects. No CGG-related accuracy correlations were found. In the visual word paradigm, CGG repeats showed no correlation with results. In picture description, higher CGG repeats correlated with disfluency measures like pauses and typical dysfluencies, while syllables and words per minute showed negative correlations as repeat size increased, indicating that as the CGG repeat size increases the syllables and words per minute decreases.

Other demographic factors

Gender and the number of languages spoken significantly correlated with RTs in picture naming and visual world paradigm tasks among FXPCs, with males showing longer RTs and multilingual participants having faster RTs. Conversely, medical history and medication use did not significantly impact performance in these tasks. Accuracy was correlated with languages potentially indicating that multilingualism yielded more errors, and with gender where males seem to produce fewer errors in objects but more in actions than female. For the CP group, RTs were correlated with medications, languages in the VWP task, where multilingualism seems to elicit faster responses, and the use of psychoactive medications seems to elicit slower RTs. No significant correlations were found between demographic factors and accuracy suggesting limited correlations between these variables and language performance.

Latency and accuracy per word and psycholinguistic factors

To summarise, for the FXPCs group in the objects and in the action condition, strong positive correlations were found between average RTs per trial and percentage of errors, indicating that increased RTs correspond to higher errors. This pattern was consistent across in-person and online subgroups. Similarly, the CP group showed strong correlations overall and within subgroups. Concerning psycholinguistic factors, in the picture naming task especially name agreement, showed significant correlations across groups, while other variables like frequency and age of acquisition had weaker effects. These findings suggest name agreement as a key factor in linguistic performance differences. In the visual word paradigm frequency emerged as the main factor influencing performance especially in the CP group, while no correlations were found in the FXPCs group in the object condition, while in the action condition name agreement emerged as a factor influencing performance across groups. Overall, these findings suggest that certain psycholinguistics factors (frequency in the object condition and name agreement in the action condition) significantly correlate with VWP performance and RTs in both FXPCs and CP groups. Notably, the correlations found in the CP group were more consistent across modalities and conditions compared to the ones found in the FXPCs group, potentially indicating different cognitive strategies.

2.5 Discussion

In Chapter 2 we outlined details of the first experiment, carried out to answer our first research question:

- a) Is it possible to identify potential differences between FXPCs and a control group through language assessment that could indicate prodromal signs of cognitive alterations in FXPCs?

Here we demonstrate that it is possible to find distinct patterns between the two groups using three non-standardised language tasks.

In the first task, the picture naming, we assessed both latency (RTs) and accuracy (percentage of errors). In analysis of latency, we hypothesised that the FXPCs group would be slower than the CP group particularly in actions compared to objects, similarly to what has been found in literature in PD patients, with similar tasks [105, 133, 174]. We did find a group effect, which partially confirmed our hypothesis of longer RTs in the FXPCs group compared to the CP group. This difference was present in the automatic scoring modality both in actions and objects. It was not present in the manual scoring modality, however the difference between groups in the object condition was trending to significance and the uncorrected significance may suggest a trend where the FXPCs group is slower than the CP group in objects compared to actions. This potential trend is present both in automatic modality, where the difference was significant, and in the manual scoring modality, where it narrowly missed significance. However, this result lacks to reach significance, therefore this explanation is only speculative, and further studies are needed to confirm it. No scoring modality effect was present. This finding validates the use of the techniques used and of the algorithm. We used two different scoring modalities because we wanted to investigate whether it is possible to automatise the analysis as much as possible to reduce the investigator intervention. Literature comparing different modalities of RTs detection is not unanimous. Some studies used both manual voice onset detection and automatic [251], others suggest the use of automatic tools [252] to reduce the investigator bias and intervention, and to standardise the procedure. We hypothesised that scoring modality would not yield different results, and our findings confirmed this hypothesis. However, some different results were present in the automatic and manual scoring modalities, where significant differences were found between groups in both actions and objects in the

automatic scoring modalities, and not in the manual. These differences may be attributed to the specific the design of the study, including both in-person versus online formats. While algorithms for detecting onset times are generally accurate, we hypothesise that the dual modality employed in our experimental design may limit the efficacy of automatic detection in this context. A considerable challenge arises in controlling for the accuracy of voice detection, particularly in the online modality. Various confounding variables, such as the sound quality in participants' environments, complicate this control and the correct detection of voice onset. Moreover, it remains uncertain whether the automatic detection algorithm effectively identifies the actual onset of a word, as opposed to considering extraneous factors, including background noise and accidental vocalisations before the target name, and it has been demonstrated that automatic tools for voice onset detection are unable to differentiate other kinds of vocalisations [251]. Additionally, a higher number of missing data points was present in the automatic scoring modality compared to the manual one, due to inability of the algorithm to detect voice onset times in some cases. In contrast, the manual scoring modality allows to mitigate inaccuracy in capturing voice onset time. Because no difference in modalities was found in CP objects and in FXPCs objects and actions, we believe that the algorithm for voice onset detection might be valid, but it is not indicated for the particular design of this study especially in the online modality for the reasons stated above. Therefore, we believe the manual detection was more accurate to be used for subsequent analysis of this study. We found an additional effect of word class across groups and scoring modalities. This confirms previous findings in literature where actions RTs were slower compared to objects RTs [231], and indicating that despite the effort in matching psycholinguistic variables, actions still seem to be inherently more complex and require more time to be recognised and named [231]. This is compatible with theories of lexical access believing in distinction that is visible at a lexical stage (lexical view) and that grammatical and semantic aspects are retrieved at the same time, corresponding to a total neural segregation (lexicalist view), and with the emergentist view which believe in a partial separation, due to a distinction between objects and actions, rather than a mere grammatical distinction [233]. However, we did not directly investigate neural network activation because it was beyond the scope of this study, therefore our hypothesis is only speculative, and further studies are needed

to confirm it. To summarise, our findings show a group difference only in the automatic scoring modality in the action condition, indicating that FXPCs are slower compared to CP. This result would put the FXPCs group in alignment with previous findings in literature on PD and other parkinsonian disorders such as CBS and PSP [174], potentially indicating a BG network involvement in actions deficit. However, we argued that potentially the automatic scoring modality might not be the most suitable in this case due to the study design, and that across scoring modality there is a difference in objects, indicating that the FXPCs group appears to be slower compared to CP group, that however was only significant in the automatic scoring modality and did not survive FDR corrections for multiple comparison in the manual modality, but was potentially trending to significance. Because it was not significant we are only speculating that if confirmed by further studies, this potentially could indicate a different pattern between FXPCs and PD patients that could help distinguish the two populations, indicating potentially different mechanisms underlying the different word class disadvantages, objects for FXPCs and actions for PD. We have shown that the PD deficit in action is believed to be connected to the BG network, while a deficit in object naming, as seen in AD, might be related to temporal lobe deficits, a network recruited by semantic memory processes [151-153]. Deficits in temporal lobe activation in FXPCs compared to controls, particularly in the temporoparietal junction (TPJ), have been already found in literature, supporting the findings pointing to objects retrieval impairment in this population [120]. Additionally, in the same study they found that elevated levels of *fMRI* mRNA predicted the reduced activation in the TPJ, potentially explaining the nature of the deficits. Given the variability in our results further studies are needed to confirm it.

We additionally investigated potential differences in task administration modality (in-person and online) to verify influence of the modality on our main hypothesis of a difference between groups. We hypothesised that the modality would affect the outcome, and our results confirmed this hypothesis as we found a modality of task administration effect, while no group effect was present. Particularly, the online modality yielded higher RTs across groups and conditions, confirming the potential presence of more confounding variables online and therefore that the two modalities affected the results. A larger sample

size is probably needed, comparing the different modalities of task administration to confirm these results.

The next step in the analysis to answer our first research question was to investigate potential differences between groups in accuracy. We hypothesised that the FXPCs group would show a higher percentage of errors, particularly disfluencies type, compared to the CP group, as seen in literature [7]. A group effect was present indicating different accuracy levels between FXPCs and CP group, however no significant differences were found in the post hoc comparison. We further investigated this aspect including mistaken answers only, and again a group effect was found that was not significant in post hoc comparison, and a modality effect was found that indicated in post hoc comparison that the FXPCs group was less accurate in-person compared to the online modality. We believe this finding can shed a light on one of the differences in modality of task administration, potentially indicating that the FXPCs group felt a higher level of stress in-person, being subject to a high level of social anxiety [26], inducing them to be less accurate, even if faster in RTs as indicated by analysis of latency. Potentially the small sample size did not allow to detect a difference in post hoc comparison, therefore further studies are needed to investigate this aspect.

To further examine group differences in accuracy, we conducted a qualitative analysis of participants' error types, and we anticipated different error patterns between groups. In contrast with our expectations, both groups showed similar error patterns across object and action conditions, with disfluencies being the most common (50% for CP, 62% for FXPCs). The main difference between group is found in the subtypes of errors, where FXPCs group had more circumlocutions, while the CP group showed more planning errors and semantic coordinate subtype errors. In the action condition, the CP group made more visual errors, especially misperceptions, which are not related to semantic but more to difficulty processing verbs [115], as well as issues in thematic and superordinate semantic types linked to semantic memory. The prevalence of circumlocutions aligns with prior research on neurodegenerative disorders, particularly in PD [132], suggesting challenges in accessing object representations in semantic memory, which can lead to the use of periphrasis or circumlocution as a strategy to express the concept [253].

Separate analyses by modality revealed a different pattern from the overall analysis in the in-person modality, in the object condition, where the CP in-person group showed more semantic errors (52%) compared to FXPCs (43%), potentially indicating a generalised difficulty in retrieving the object words, while FXPCs had higher disfluencies, particularly evident in planning, circumlocution, and hesitation and omission/grammatical errors, pointing to compensatory strategies to access the target word, and the use of less informative speech. In the action condition, visual errors were most frequent for the CP group, likely due to picture interpretation difficulties, suggesting that despite matching the psycholinguistic variable, actions are probably inherently more difficult, with disfluencies and semantic errors following. Comparing modalities, in-person participants demonstrated more semantic errors, especially in the CP group, while online CP participants had more planning errors, likely due to task administration differences rather than inherent language deficits as it is not higher in the in-person group. Planning errors mainly involve filled pauses and it is a very common kind of disfluent language marker [253], but because it is only present in one of the modalities it can maybe be related to a higher level of distraction of the online group and the necessity of strategies to delay the retrieval of the specific word. In the FXPCs group, in-person participants struggled more with semantic, visual, omission, and grammatical errors, whereas online participants showed increased article revisions and circumlocutions within disfluencies.

In the second linguistic task, the visual word paradigm, we again hypothesised differences between groups in language reception this time. The results confirmed the presence of a main group effect, that did not survive FDR correction in post hoc comparison, which however revealed a potential trend to significance showing slower RTs for the FXPCs group in both objects and actions. This potentially confirms the trend found in the picture naming task indicating a disadvantage for FXPCs compared to the CP group, however these results lack to reach significance therefore further studies are needed to confirm this trend. Additionally, a word class effect was again present across groups, confirming actions retrieval more challenging than object retrieval even in language reception. Furthermore, we hypothesised a difference in modality in this task as well, and the results of the next comparison including modality as a factor confirmed that the FXPCs group in the action condition had slower RTs

in the online modality compared to the in-person one. While the word class effect was again confirmed, the group effect was not present in this case confirming the influence of modality of task administration on the main hypothesis of a group difference.

The final step in addressing our first research question was to investigate group differences in the third linguistic task, the picture description, where we hypothesised the presence of a more disfluent speech pattern in the FXPCs group. We observed no significant statistical differences between the in-person and online modalities, prompting us to combine both for the first analysis. Our hypothesis was confirmed as the FXPCs group performed worse than the CP group in several disfluency measures, including, blocking, total number of disfluencies, both typical and stuttering like, phrase revisions and word revisions (Table 11). This finding is consistent with other findings in the literature where the FXPCs group produced more disfluencies than control group [7]. Previous studies detecting disfluent language patterns in FXPCs were mainly based on 5 minutes of expressive language sample collection. Here we show, that even with a more structured and much shorter task like a picture description, which can be administered both in-person and online, different language patterns can be identified, with analysis that can be automated using designated programs. Subsequently, we analysed the group's general language performance through a CLAN program specifically designed for dementia and aphasia [245], which we considered particularly appropriate to our sample. Here we hypothesised the presence of a richer language pattern in the CP group, that would confirm the previous result of a more disfluent speech pattern in the FXPCs group. The results confirmed this hypothesis as the CP group produced a significant higher number of adjectives compared to the FXPCs group. This measure indicates a richer, more complex and descriptive language, as adjectives are supplementary and non-primary part of speech, used to add extra information to the speech. Furthermore, the FXPCs group produced a significantly higher percentage of retracing (percentage of self-corrections or changes) compared to the CP group, indicating more hesitant and disorganised speech, revealing potential difficulties in speech planning. Difficulties in planning are usually related to executive function deficits, which are commonly found in individuals with FXTAS, therefore this could be an indication of executive function deficits as a prodromal sign of cognitive decline, or could indicate that even without FXTAS certain

individuals could show linguistic deficits [7]. Together, these findings portray two distinct language patterns between the groups: the control group demonstrates a richer and more complex descriptive language, while the FXPCs group shows a more disfluent and disorganised pattern, consistent with previous findings in the literature [8].

We then addressed our second research question:

b) What is the nature of language disfluency patterns in FXPCs and are these related to a primary language deficit?

We hypothesised that the disfluencies identified in the FXPCs group could be originated from a primary language deficit expressed in the form of lexical access difficulties. Disfluencies in language are usually correlated to executive function deficits, often linked to planning and organisation; however, disfluency in this population might not stem from those deficits, as several studies failed in finding a link between the disfluencies and the executive disfunctions, typically found in this population [8]. To tackle this, we correlated the disfluencies observed in the picture description task, with the RTs in the picture naming and visual word paradigm task, which tap directly into lexical access. Our hypothesis was confirmed as the disfluencies identified in the FXPCs group are associated with slower RTs particularly in language production. This finding suggests a potential impairment in lexical retrieval, where delays in word retrieval may contribute to disfluent speech patterns in this population. Interestingly, in a very recent study [109] comparing language performance of FXTAS symptomatic males and females, the authors found several language predictors of symptom severity, including executive dysfunction. Particularly, executive functioning was predicted by lexical diversity, dysfluency, and speech rate, potentially differentiating the FXPCs disfluent language profile from the FXTAS profile, which is indeed connected to executive dysfunction. Additionally, in their research like in our study the disfluencies rate did not increase with age [109]. These findings are particularly interesting in the debate about the nature of language deficits, and whether language deficits are influenced by other cognitive structures, or it is *sui generis*. In this study, our findings connecting disfluencies to a language specific process (lexical access), seems to corroborate the second hypothesis. Lexical access involves areas such as the inferior frontal gyrus which together with the cerebellum are involved in lexical

representation [201], and because the cerebellum is one of the area most affected in FXTAS, we believe that this finding is particularly relevant. However, we did not directly investigate correlations with executive functions, here, therefore these findings need to be interpreted with caution and further investigated in future studies.

Finally, we performed some exploratory correlation analysis to investigate potential demographic factors affecting performance. Age was correlated in the picture naming task with RTs and accuracy in the CP group in the action condition, but not with the FXPCs group in RTs, and negatively with FXPCs in accuracy in the object condition. In the visual word paradigm age was positively correlated with RTs in both groups, while in the picture description task the CP group showed an increase in disfluencies with age, while the opposite pattern was present in the FXPCs group. An increase in word retrieval time is supported by literature on healthy aging [126], suggesting that changes involving the aging brain might have an influence on word retrieval speed from the mental lexicon. Interestingly this influence is not present within the FXPCs population, meaning that their performance in language speed is probably not primarily correlated with age. Notably, previous studies in literature found a correlation with age and disfluencies in FXPCs women, which was not found in this study, in the picture naming task [7] [108]. However, neither of those studies investigated disfluencies in a picture naming task, whose different setting might influence performance. Additionally, the inverse relationship found in the FXPCs group is consistent too with literature on healthy aging indicating that compensatory strategies might be in place as we get older, to control for speech accuracy [126]. However, this is in contrast with prior studies which linked disfluent speech patterns to aging in the FXPCs population [108]. This result also might show that the higher percentage of errors in the FXPCs group revealed by the qualitative analysis is not age-related but probably influenced by other factors. On the contrary, the correlation observed in the CP group, potentially indicates that the CP group performance in accuracy in the action condition seems to get poorer with age, as shown in the RTs correlations. Taken together these findings potentially indicate that different mechanisms might be in place for CP and FXPCs, and between objects and actions. In the action condition of the CP group, word retrieval speed decreases with age, and accuracy becomes poorer in older individuals. This pattern, that is not present in the object condition, potentially

indicates that action retrieval impose greater challenge in aging population, while object retrieval is not particularly affected by age. Interestingly, in the FXPCs population, different mechanisms are potentially in place, and while age is not a potential variable that can affect performance in the picture naming task, other variables might be. However, further investigation is needed to explore these findings as the correlations were not strong enough to draw definitive conclusions. While no correlations were found in the visual word paradigm and in accuracy in picture naming, the number of CGG repeats was correlated with RTs in the picture naming task where a greater CGG repeats size was associated with slower RTs in the object condition. Correlations were also found in the picture description task indicating that a higher number of CGG repeat size was correlated positively with a higher number of certain types of disfluencies, and negatively with other types of disfluencies such as words and syllables per minutes, indicating a decrease of words and syllables per minutes as the CGG repeat size increases. Associations with CGG repeats size have been found in previous studies, however literature on the field is not consistent. A curvilinear association between language abilities and CGG repeat size was identified in previous studies [8]. However, those findings suggest a correlation with disfluencies in speech, which is higher at lower and mid-levels of repeat size, while our study suggests a correlation with speed which becomes slower as the CGG repeat expansions increases, and no correlation with disfluencies and accuracy level in general, as other studies suggested too [108]. Although unfortunately the number of data we have is small, it is still interesting that there is indication of a potential correlation that might help finding potential reasons for individual differences in the FXPCs group. Other demographic factors correlated with participants' performance in the FXPCs group were gender, with males showing longer RTs in both picture naming and visual word paradigm, but fewer errors in accuracy, and the number of languages spoken, where multilingual participants had faster RTs in both production and reception, but a higher percentage of errors. In the CP group, no correlations were present in the picture naming tasks, while in the visual word paradigm multilingual participants showed again faster RTs, and history of medications also showed a significant correlation where the use of psychoactive drugs elicited slower RTs. This correlation seems to point to a disadvantage of males over females in latency, which is consistent with a higher severity of FXTAS symptoms in males

[254], while in terms of languages, the higher number of languages spoken does not seem to delay words' recognition, instead multilingualism may play a role in enhancing certain cognitive mechanisms (e.g. executive function, working memory) necessary for word retrieval, improving performance in tasks requiring naming of objects or actions, as seen in literature [255]. Concerning accuracy, in the object condition females had a higher percentage of errors and in the action condition the pattern is opposite, potentially indicating a different language pattern for males and females. However, the number of males who entered this study is too small to draw a significant conclusion. Multilingualism also yielded correlations, as in this case a higher percentage of errors seems to be correlated to the higher number of languages spoken in the FXPCs group. Therefore, it looks like multilingualism does not affect how fast a word is retrieved in the population considered, but it does correlate with accuracy in the FXPCs group. This finding is consistent with literature on multilingualism, showing that while speaking more than one language can improve executive functions abilities, it can also impact the word retrieval process, because of the different languages competing for retrieval of the correct word [256]. In the CP group, the medications seem to contribute to delays in RTs in the VWP, particularly the psychoactive drugs seem to be associated with slower RTs, a finding consistent with impaired cognitive performance due to psychoactive substances [257]. Lastly, we investigated the presence of correlations between latency and accuracy for each word, to assess whether the words that were named slower were also the most difficult. The correlation analysis confirmed this hypothesis as a strong correlation between RTs and accuracy was found across groups and modalities, meaning that RTs significantly correlated with accuracy per word, and that potentially some inherent challenges of the words were causing difficulties in accessing the lexical item. To further investigate this aspect, we wanted to explore which psycholinguistic factors that were used to match each trial between objects and actions correlated with performance. Name agreement emerged as the main variable influencing performance across groups and word class in the picture naming task, while other variables such as frequency and age of acquisition showed weaker effects. In the visual word paradigm in object condition of the CP group word frequency was negatively correlated with RTs, while name agreement was consistently negatively correlated with RTs in the action condition, across groups and modalities. These

differences between the language production and reception task and between objects and actions, suggests that unique mechanisms and factors influence lexical retrieval in production and reception and in different word classes. Name agreement emerges as the main psycholinguistic factor potentially involved in lexical retrieval performance, particularly in action processing. This variable represent the degree to which different participants use the same name to identify a picture and it potentially affects some of the sequential stages involved in picture naming, such as object recognition and lexical selection, as in low name agreement a single item can activate multiple concepts instead of just one, resulting in delays or errors in retrieving the target word, as shown in the literature[258]. Here we confirm the importance of this variable across groups, particularly in the action condition, which was more challenging potentially because of the higher difficulty of attributing one single name to the action depicted. This exploratory correlation analysis in the linguistic tasks shows some relationships between variables, however these are not always strong enough to establish a certain relationship between the variables, so we cannot conclude that these factors impact language performance unequivocally. However, some consistent correlation, for example the one between age and most linguistic measures in the CP group might suggest trends aligned with healthy aging strategies, as noted earlier. In the FXPCs group, however, additional factors, including a high prevalence of comorbidities like FXTAS and FXAND, also need to be considered.

Chapter 3: Towards the assembly of a complex candidate biomarker: combining molecular and linguistic measures.

3.1. Introduction:

In this chapter, we will answer our research questions 3 and 4 (3. is it possible to identify potential alterations in plasma-based proteins in FXPCs and in FXS? Are these alterations similar? 4. Is it possible to find links between linguistic and molecular measures by combining molecular and linguistic measures to advance development of treatments for FXPCs?). For question 3, we hypothesised that FXPCs and FXS patients have altered expression of plasma-based proteins compared to controls and we describe here two experiments assessing molecular measures we performed to investigate this aspect. First, we will provide an overview of the rationale for analysis of the same plasma-based proteins in FXS and FXPCs. We will then describe the methodology and results of the molecular analysis carried out on FXPCs samples (Molecular Experiment 1), followed by the methodology and results for FXS samples (Molecular Experiment 2). For question 4, we hypothesized that molecular and linguistic measures can be combined together to show a correlation between the two measures, and here we explore the potential correlation between molecular and linguistic analysis, with the goal of potentially finding correlations that could advance biomarker discovery in these populations. Linguistic assessment measures were only available for FXPCs, preventing combined molecular and linguistic analysis for FXS. Finally, we will discuss the findings from both molecular experiments and the broader implications of integrating molecular and linguistic assessments.

3.1.1 The potential for investigation of Microtubules Dynamics (MT) dysregulation as a therapeutic target for FXS and Fragile x Premutation Carriers.

We described in Chapter 1 the role of Microtubule dynamics in neuronal plasticity and how measuring post-translational modifications (PTMS) can provide insights into how MT regulate formation and maintenance of the polarised nature of neurons. Acetylation and tyrosination are important as they represent expression of α -tubulin isoforms, acetylated and tyrosinated α -tubulin

that are considered markers of MT dynamics. As mentioned in 1.5, acetylated α -tubulin is associated with less dynamic microtubules, while tyrosinated α -tubulin correspond to a more dynamic state.

Markers of altered MT have been found in depression models and in neurodevelopmental disorders. Notably similar alterations have been observed in rodent models both in the brain and in plasma samples, indicating that expressions of these proteins in the plasma can be potentially indicative of expression of the same proteins in the brain [190]. In this study, we analysed α -tubulin PTMs in plasma samples from both FXS patients, and FXPCs.

We believe that investigating potential markers of alterations in MT in plasma samples of individuals with different kinds of mutation on the *FMRI* gene could be important not only because previous studies have shown that similar alterations can happen in plasma and in the brain, but also because a potential dysregulation can be indicative of the general health status of the body.

Microtubules are fundamental in neurodevelopment as they are important for neuronal development and growth, therefore, dysregulation of MT can have serious consequences on neurodevelopment. However, because of the many fundamental roles played by microtubules, dysregulated microtubules dynamic has been shown to be present in several neurodegenerative disorders too.

FXS and FXTAS are caused by two different kinds of mutations on the same gene *FMRI*, which encodes the FMRP protein. FMRP, interacts with microtubule-associated proteins such as 1B (MAP1B) and its loss is believed to lead to dysregulated microtubule-related mechanisms, hence potentially disrupting microtubule dynamics [259]. FXS is characterised by loss of FMRP production because expansion of the CGG trinucleotide over < 200 repeats causes methylation which in turns silences the *FMRI* gene [260].

However, decrease in the production of FMRP has been found in FXPCs as well, probably due to the increased transcriptional activity which causes inefficient translation resulting in decreased levels of FMRP production [260, 261]. This suggests that, although the molecular mechanisms differ, both FXS and FXPCs, may involve disruption in FMRP-related pathways, potentially affecting microtubule dynamics and neuronal function.

Many of the functions of FMRP are yet to be uncovered, however we know that is fundamental in brain development and in synaptic plasticity throughout the lifespan [262]. FMRP is also a mRNA-binding protein which is believed to play

important roles in regulation of trafficking in dendrites, and FMRP granules have been shown to interact with *MAP1B* mRNA, which encodes a microtubules-associate protein 1B^{xxviii}, a FMRP target [263]. Interestingly, in a study with cultured hippocampal neurons levels of FMRP in dendrites have been shown to be affected by microtubules dysregulation [263]. MAP1B is a known regulator of MT dynamics and stability, and it regulates its activity through phosphorylation. This process involves several protein kinases including glycogen synthase kinase 3 (GSK-3), which has two paralogs GSK α and GSK β , encoded by different genes [264]. GSK-3 is considered to be an important regulator of axon growth and branching, and its activity is regulated by phosphorylation. This kinase is an upstream regulator of microtubules in neurons, and it is believed to mediate phosphorylation of MAP1B influencing its activity. This regulation plays a crucial role in processes such as neurite branching which requires microtubules reorganisation [265]. One of the known effects of reducing FMRP levels is that inhibitory phosphorylation of GSK-3 is reduced in in-vivo models of FXS [264]. Interestingly, phosphorylation can be modulated pharmacologically. Lithium citrate has been shown to increase phosphorylation in-vivo [264], and to reduce hyperlocomotion in *Fmr1* KO mice [266], and to improve animal behavioural deficits in animal models [267]. Additionally, lithium was tested in an open-label trials on FXS individuals and it was shown to have some positive effects on symptoms [268]. In recent years several new GSK-3 inhibitors are being explored for neurological disorders with promising results, particularly targeting GSK-3 β , a Tau regulatory kinase, that influences microtubule binding and together with FMRP interacts with other microtubule associated proteins [263, 269-271]. These new inhibitors have been shown to potentially improve symptoms in in-vivo studies, for examples showing a protective effect against seizures, improvement on cognition, and several behavioural phenotypes in *Fmr1* KO mice [272-274]. These new therapeutic targets could be important to advance drug discovery in FXS, but we believe it is important to investigate potential markers of dysregulated MT dynamics in the FXPCs population as well.

^{xxviii} The role of microtubule associated proteins (MAP) will be further discussed in the next paragraphs of this chapter.

3.2 Molecular Experiment 1: Fragile X Premutation Carriers

3.2.1 Background

In addition to the loss of FMRP experienced by some of the FXPCs, most clinical symptoms experienced by premutation carriers are usually associated with the presence of RNA toxicity due to elevated levels of *FMR1* mRNA [1]. This increase is approximately proportional to the CGG repeat size and causes a defective translational efficacy of *FMR1* mRNA, which leads to a toxic cell environment [275]. Since mRNAs are transported along microtubules (MTs) via active motor-driven mechanisms, while the underlying essential factors and dynamic interactions are largely unknown, this could provide further rationale for investigating MT dynamics. Understanding these processes could lead to important findings in terms of uncovering pathological mechanisms that could be targeted for new therapies. Furthermore, dysregulation in MT dynamics has been found in several neurodegenerative disorders: because of the risk of developing cognitive decline and FXTAS in premutation carriers, we believe targeting investigation of MT dynamics in FXPCs as well as in FXS, could be important not only to better understand the underlying mechanisms of the conditions associated to the premutation, but also to further explore the possibility of finding new candidate biomarkers of disease. In the next section we will give an overview of findings on the mechanisms underlying MT dynamics dysregulation in neurodegeneration.

Dysregulation of Microtubule Dynamics (MT) in neurodegeneration

Neurodegeneration refers to the gradual decline of neuronal functions or structures, often starting in the axons. As discussed in Chapter 2, many neurodegenerative diseases exhibit common clinical symptoms and share pathological features, including alterations in microtubules [276]. The complex system which regulates microtubules is composed by the perfect balance of several different aspects. An important characteristic of microtubules, shown in chapter 1, is “dynamic instability”. This term refers to their ability of being dynamic, alternating phases of growth and shrinkage. Usually both dynamic and stable microtubules coexist in neurons and other cells, and several different proteins help maintain the balance between the number of stable and dynamic microtubules. Among these, proteins such as non-hydrolysed GTP tubulin subunits, are thought to stabilise the typically more dynamic plus end of

microtubules. Additionally, plus-end tracking proteins (+TIPs) play a crucial role in various mechanisms of microtubule dynamics, including interactions with other structures [276]. Length of microtubules is also another important aspect that contributes to the overall system, and a healthy neuron contains both short and long microtubules. This equilibrium is regulated by enzymes, called Microtubule-severing-enzymes, that can cut microtubules' length when necessary, but also contributes to the amplification of the microtubule cytoskeleton, as promoters of microtubules growth (particularly, this contribution on microtubule growth has been shown in Katanin and Spastin [277]). In general, the complex microtubular system is regulated by an arsenal of proteins, called microtubule associated proteins (MAPs) as we have seen in the introductory section 3.1. Some of those are important regulators of the Microtubule-severing-enzymes. One of these MAPs is the protein Tau, which is fundamental for maintaining cytoskeleton stability and dynamics. Tau protein is encoded by the MAPT gene and interacts with microtubules through electrostatic interactions between the positively and negatively charged regions of the tubulin [278]. One of the reasons why microtubules are believed to play an important role in neurodegenerative diseases is their fundamental transportation role, and it has been shown that many pathologies connected to Tau show compromised axonal transport [279].^{xxix} Moreover, microtubules provide many other fundamental functions for the cells in general and the neurons in particular, including regulating neuronal polarity and morphology, therefore a dysregulation in microtubules dynamics could potentially highly contribute to neurodegeneration through different aspects. As a result targeting microtubules with therapeutic interventions could be helpful to help slow progression of neurodegenerative diseases [276]. Another important aspect influencing microtubule stability and dynamics is the post translational modifications of the microtubule's subunits α and β tubulins.

Tubulin post-translational modifications (PTMs)

Both subunits α and β tubulins- are modified by certain post-translational modifications (PTMs) that are markers of microtubule's stability. Some of these modifications are acetylation and tyrosination. A stable microtubule is usually

^{xxix} This aspect will be further discussed in section 1.5 dedicated to post-translational modifications.

acetylated and detyrosinated. If the balance is dysregulated, this can cause defects in microtubules functions as the modifications themselves influences microtubules stability, making them too stable or too dynamic [280]. Several modifications can occur at the same time and play an important role in regulating binding to a high number of proteins, hence contributing to the overall neuronal cytoskeletal dynamics. We will now briefly describe the two PTMs we will consider in this study.

Tyrosination and Acetylation.

Tyrosination is characterised by the incorporation of tyrosine in the α -tubulin C-terminal. α -tubulin is tyrosinated at the beginning and then it goes through a cycle of addition and removal of the tyrosine, a phenomenon called detyrosination. Tyrosination is very important for the microtubule's assembly state, as assembling microtubules are only composed by tyrosinated tubulin, therefore the assembly phase is strongly dependent on tyrosination, and newly formed tyrosinated microtubules are very dynamic. Detyrosination instead seems to play an important role in associating with motor proteins (Kinesin-1 is an example of a protein which binds preferably to detyrosinated microtubules in neurons), and typically detyrosinated microtubules are more stable [281]. Typically, neurons are composed by a higher number of detyrosinated microtubules, as they are important to direct the movement of motor proteins to the different neuronal compartments, for example Kinesin 1 which is involved in mitochondrial trafficking, moves preferable on detyrosinated microtubules, as well detyrosinated microtubules are believed to play a role in severing enzymes, which play a fundamental role in axonal growth, trafficking and branching [281]. Detyrosination is associated with increased microtubule stability, although it is believed to be not sufficient to ensure regulation of microtubule dynamics as some findings suggest that tyrosination is also a key mechanism to regulate protein interactions in microtubule [282]. Therefore, the tyrosination/detyrosination cycle interlinks with microtubule dynamics and has been implicated as a fundamental mechanism for learning and memory consolidation, and its dysregulation potentially plays a role in synaptic plasticity and dementia [283, 284]. Acetylation happens on lysine 40 (K40), and it is considered very important for protein-protein interactions within microtubules, and it is also important to prevent mechanical damage to the microtubule as it

enhances flexibility [285]. Acetylation has been one of the most studied tubulin PTMs and one of the most puzzling: many mechanisms concerning the role played by acetylation are yet to be understood. Acetylation plays an important role in preserving the mechanical structure, and additionally, this PTM is believed to play a role in MT dynamic by regulating self-assembly rates and MT severing proteins [286]. Acetylation as well as tyrosination, is also believed to regulate activity of motor proteins, as for example loss of α -tubulin acetylation is believed to modify binding and trafficking of motor proteins in *in vitro* studies. Additionally, it is believed to be important in axonal growth and neuronal plasticity as increased levels of acetylated α -tubulin have been observed during axonogenesis [282]. However, when the level of acetylated tubulins is too high this can mean an imbalance in the microtubular system. Acetylated tubulin is generally considered a hallmark of stable MT. However, findings about the role of acetylation in MT dynamics are inconsistent and, despite clearly playing an important role in the microtubular system, a clear picture is still not available [286]. Therefore, levels of α -tubulin isoforms, such as acetylated and tyrosinated α -tubulin, could be a marker of regulation of MT dynamics, and measuring those levels could help as understand potential dysregulated mechanisms in the complex microtubular systems [287]. As discussed in Chapter 1, there has been growing interest in identifying alternative, less invasive fluid biomarkers beyond cerebrospinal fluid (CSF). Since α -tubulin isoforms are detectable in plasma, they may offer a valuable tool for investigating potential drug targets and assessing overall physiological health.

Reduced MT stability in neurodegeneration.

Several mechanisms involved in the complex microtubular structure and dynamics could contribute to neurodegeneration, here we briefly give an overview of some of them as reported in literature. Neurons are mainly composed by stable microtubules as it is an important feature for neuronal morphology, but dynamic pool of microtubules are also important for example for regeneration of neurons and turnover in general. In several studies hyper stabilised microtubules have been shown to contribute to neurodegeneration, because they can compromise or reduce axonal transport, or can trigger neuronal detriment [276]. Microtubule dynamics is fundamental to maintain healthy neurons, particularly in some neurodegenerative disorders associated with

memory loss. To successfully enhance memory formation microtubules need to be dynamic to allow synaptic plasticity [288]. Since memory loss typically appears in the early stages of neurodegenerative diseases, it is thought that dysregulated microtubule dynamics could help identify the risk of neurodegeneration from these initial stages [289]. Microtubules are fundamental for synaptic plasticity, therefore, because of all the above-mentioned important roles that microtubules have, it is not surprising that changes in the microtubule system are seen in many neurodegenerative disorders. Axonal transport is very important for synaptic plasticity and in general neuronal activities. Microtubules form sort of railway where all kinds of organelles, vesicles and proteins can move, particularly in neurons. Because of the specific conformation of their long axons and dendrites, there is a need for everything to be perfectly organised in microtubule functions, therefore when there is a dysregulation in microtubules activities this can be correlated to axonal transport defects and this has been associated with early neurodegeneration [282]. As mentioned in the previous section, Tau protein is a very important MAP because it binds to axonal microtubules and its main function is to promote microtubule assembly by tubulin and to regulate stability through several different mechanisms, as well as promoting regulation of folding and aggregation, spacing and diameter [290]. It largely contributes to axonal transport as well and its PTMs, particularly acetylation and phosphorylation, plays a regulatory role on microtubule stability [282]. In AD patients' several findings show a lack of Tau acetylation, which increases phosphorylation, causing microtubules dysregulation [291]. Hyperphosphorylated Tau has been found in other neurodegenerative diseases, including PD, where Lewy Bodies proteins contains both Tau and α -synuclein as shown in Chapter 1. Accumulation of the latter is a typical hallmark of PD, but knowledge of neuronal α -synuclein functions is still partial. However, it is clear that α -synuclein interacts with tubulin subunits, and it is believed to promote microtubules polymerisation and axonal growth rate [292]. Several other PD-associated proteins are believed to have a role in regulation of microtubule dynamics, which can affect axonal transport, synaptic plasticity and eventually neurodegeneration [282]. AD and other neurodegenerative disorders are characterised by the presence of neurofibrillary tangles (NFTs) composed by modified Tau proteins [293]. Because Tau contributes to axonal growth and neuronal morphology as mentioned above, neurodegeneration can be caused by

a dysregulation in axonal transport [294] and some pathological aspects of neurodegenerative disease can be directly related to dysregulation in microtubules stability[295] .Another contribution to the study of the connections between MT and neurodegeneration is the investigation of MAPT mutations. Some of them have been described in frontotemporal dementia (FTD) with Parkinsonism, and progressive supranuclear palsy (PSP), which are believed to contribute to the link between MT dynamics and neurodegenerative diseases [296, 297] . As we have seen in Chapter 1 and 2, FXTAS is now well recognised as a neurodegenerative disorder and symptoms involve tremor, ataxia, neuropathy, parkinsonism, cognitive changes including memory loss and executive dysfunctions [25]. In terms of brain alterations white matter changes have been documented in the middle cerebellar peduncle (MCP) and periventricular areas and the splenium of the corpus callosum [298], as well as intranuclear inclusions in both neurons and astrocytes [299], and additionally parkinsonian features including loss of dopamine cells and Lewy-body inclusions [300]. Dementia is usually developed by 50% of males with FXTAS and it is less common in females. Interestingly, several medical conditions including neuropathy, hypertension, tremor and neuropsychological issues have been documented in carriers without FXTAS as well. It is likely that many of those symptoms and pathophysiological features can happen well before the development of FXTAS [34]. Given these findings, investigating potential dysregulation of MT dynamics in plasma samples from FXPCs could provide valuable insights into early alterations, potentially serving as indicators of broader systemic health status.

Neurofilament Light Chain (NfL) as a candidate biomarker

As mentioned in 1.5 another protein that can be detectable in both CSF and plasma samples [301], is Neurofilament light chains (NfL) in which is considered a key biomarker in neurodegeneration. NfL serves as an indicator of neuronal damage with increased levels of NfL been found in Amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD) parkinsonian disorders, and in AD, additionally increased NfL levels could be found as early as 15 years before the development of symptoms [302]. ^{xxx}Therefore, measuring levels of NfL could be a good candidate to be a prognostic biomarker for FXTAS. Despite

^{xxx} For further review of NfL in neurodegeneration see Chapter 1.

being considered a non-specific biomarker, as increased levels of NfL could be found in many neurological disorders, it has already been successfully used as a candidate for differential diagnosis between parkinsonian disorders; in a study measuring NfL levels in Parkinson Disease, and a group of Atypical parkinsonian Disorders (APD), Progressive Supranuclear Palsy (PSP), corticobasal syndrome (CBS) and multiple system atrophy (MSA) and in an early stage cohort, the authors successfully distinguished between quantification of NfL in PD and in APD, suggesting a potential for the use of NfL for differential diagnosis [303]. Measuring NfL levels could therefore be an ideal candidate to assess the status of the disorder in FXPCs and detect potential increased levels associated with neurodegeneration. In conclusion, we believe that combining quantification of α -tubulin PTMS and NfL levels within the same cohort of patients compared to controls, could contribute to advance knowledge of the overall health status of FXPCs, and potential help identify individuals at risk of developing cognitive alterations.

3.2.2 Materials and methods

Population

The population is the same considered for the in-person participants in chapter 2. The in-person participants consist of 31 participants, with a predominantly female sample (27 females and 4 males). Among them, 28 are on medication, 7 had a medical history of neurological, neurodevelopmental, or mental health disorders, and there was 1 pregnant participant. This group has an average age of 40.09 years, with a standard deviation (SD) of 13.14 and a standard error of the mean (SEM) of 2.36. The FXPCs group includes 14 female participants exclusively. Within this group, 11 were taking medication, 3 had a relevant disorder history, and 1 participant was pregnant. The mean age for this group is 48.57 years (SD = 8.89, SEM = 2.37). Lastly, the control participants (CP) group consists of 17 participants, including 13 females and 4 males, of whom 7 are on medications and 4 have a medical history of neurological or related disorders, with no pregnant participants. This group shows a lower average age of 33.11 years, with a standard deviation of 12.04 and a standard error of 2.92 (Table 23).

	Total	Females	Males	Medications	Medical history	Pregnancy	Age
In-person n	31	27	4	28	7	1	40.09 40.09,
FXPCs n	14	14	0	11	3	1	48.57 48.57,
CP n	17	13	4	7	4	0	33.11 33.11,

Table 23: Demographic characteristics of the population in Molecular Experiment 1. This table describes the population sampled in-person for molecular analysis, divided by group FXPCs and CP.

Samples collections and plasma separation

9 ml of blood samples were collected from patients in-person in Trinity College Dublin 15 minutes before performing the linguistic tasks. The PhD candidate personally collected blood samples from the control group, while a clinical nurse from the St James Clinical Research Facility collected blood samples from the FXPCs group. Plasma was then extracted following a well-established separation protocol by a member of the Ulysses Neuroscience Ltd. team.

Each sample underwent two centrifugation steps:

- **Whole blood centrifugation:** Performed within one hour of collection at 200g for 15 minutes at room temperature.
- **Platelet-rich plasma centrifugation:** Conducted at 2100g for 10 minutes at 4°C to further separate plasma.

The resulting plasma was stored at -80°C with 2% Protease Inhibitor Cocktail (PIC) until further analysis.

Analysis of α -tubulin PTMs.

Plasma protein samples preparation and analysis via infrared Western blot was done by a member of the Ulysses Neuroscience Ltd. team. Plasma total protein concentration was analysed by Bradford Assay using BSA standards and uniformly equalised to 10 mg/μl, respectively. Samples were then diluted 2x in Laemmli buffer to a concentration of 5 mg/μl before analysis by infrared Western blot for acetylated vs total α -tubulin, and tyrosinated vs de-tyrosinated (Glu) α -tubulin quantification. All Western Blot's were repeated 2 times for an n of 2 technical replicates. α -tubulin PTMs were measured as described previously

[193]. Samples were loaded at 0.15 µg to 10% bisacrylamide/trisacrylamide pre-cast gels followed by transfer to polyvinylidene fluoride (PVDF) membrane, blocking, overnight primary antibody incubation (Sigma), washing, secondary antibody incubation for 1 h (Sigma), washing and scanning on the CLx Odyssey imaging system (Li-Cor, U.S.A.). Microtubule dynamics were assessed by measuring the levels of three different α -tubulin PTMs – acetylated (Acet), tyrosinated (Tyr), and de-tyrosinated (Glu) α -tubulin. Acet was expressed as a ratio of total α -tubulin and Tyr was expressed as a ratio of Glu α -tubulin. Protein signal detection and quantification were done through the software licor Empiria Studio v2.2. first blinded to sample ID by a separate individual member of the Ulysses Neuroscience Ltd. team, then again by the PhD student, to control for consistency of results. Analysis of protein signal was performed and were obtained by normalisation of acetylated (Acet), total (Tot), tyrosinated (Tyr) and de-tyrosinated (Glu) α -tubulin signal to Transferrin (Serum Loading Control), respectively. Transferrin was used as housekeeping protein in this case. Then, the acetylated α -tubulin/transferrin signal ratio was normalised to total α -tubulin/transferrin signal ratio, and the tyrosinated α -tubulin/transferrin signal ratio to de-tyrosinated (Glu) α -tubulin/transferrin signal ratio. Finally, signal ratios were normalised to the average of the Controls Participants per gel to avoid gel to gel variation when completing the full sample groupings and for replications. Plasma from FXPCs was analysed along with plasma from CP to investigate MT dynamics in both groups. MT dynamic status was determined as a ratio between Acet/Tub-Tot/Tub (Acetylated over Total α -tubulin) and Tyr/Glu-Tot/Tub (tyrosinated over de-tyrosinated α -tubulin). Both increases in the Acet/Tub-Total/Tub ratio and decreases in the Tyr/Glu-Total/Tub ratio are associated with a decrease in MT dynamics meaning that the microtubules are hyper stabilised, while the opposite would indicate hyper accelerated MT dynamics, which would also influence MT dynamics regulation.

Electrochemiluminescent Elisa: NfL

Electrochemiluminescent Elisa was performed by a member of the Ulysses Neuroscience ltd. team. NfL levels were analysed using the R-PLEX Neurofilament L (Human; K1517XR-2) kit). Plate was washed 3 times with 150 µl of wash buffer (PBS 0.05% Tween) per well. Plasma samples were diluted

1:1 with diluent 2 (kit) and standard curve was prepared (kit). 50 µl of samples and standards were added to each well and plate was incubated at RT with shaking for 2 h. The plate was washed 3 more times. 25 µl of the detection antibody (kit) was added to each well and plate was incubated at RT with shaking for 2 h. Washing stage was repeated and 150 µl of 2X read buffer (kit) was added to each well. The plate was analysed using the MSD instrument [MB1]. All plasma samples were diluted 1:1 with kit diluent and 50 µl of samples and standards were added to each well. Plates were analysed using the MESO QuickPlex SQ 120.

Statistical analysis

To investigate potential differences between CP and FXPCs in plasma-based proteins answering the third research question (c) Is it possible to identify potential alterations in plasma-based proteins in FXPCs and in FXS? Are these alterations similar?), statistical comparisons were made using unpaired t-tests, first for each replicate and then for the average of the two replicates. Statistical analysis was performed by the PhD candidate on the raw data using licor Empiria Studio v2.2., Invivostat v4.3.0 and Grahphad Prism v. 10.2.3.

Exploratory correlation analysis

Furthermore, we performed some additional exploratory analysis to investigate effects of medications, and we performed ANOVAs to compare medicated and non-medicated FXPCs, and medicated and non-medicated CP. ANOVAs were followed by Fisher's LSD pairwise comparisons with $p < 0.05$ set as the level for significance. Statistical outliers (i.e. data points above or below 2 standard deviations from their group mean) were removed for the final analysis. Additionally, Pearson correlation was used to assess the relationship between age, CGG repeat size, and acetylated α -tubulin, tyrosinated α -tubulin and NfL in the FXPCs group, after verifying normal distribution. Spearman Rank correlation was performed to identify potential correlations between age and acetylated α -tubulin, tyrosinated α -tubulin and NfL in the CP group, and between previous medical history of neurological, neurodevelopmental and mental health disorders and acetylated α -tubulin, tyrosinated α -tubulin and NfL in both groups. All analyses were performed separately for the CP and FXPCs group. Results are presented as mean $\pm$ SEM.

3.3.1. Results

Western Blot analysis

We hypothesised that the FXPCs group would have altered expression of plasma-based proteins compared to the CP group. As shown in Figure 35 our hypothesis was confirmed in the α -tubulin PTMS, particularly when comparing plasma levels of Acet/Tub-Tot/Tub between CP and FXPCs, as the independent t-test produced an effect between groups in the second replicate ($t(20)=2.995$, $p=0.0078$) and in the average of the replicates ($t(23)=2.420$, $p=0.0238$). The FXPCs group had indeed lower levels of Acet/Tub-Tot/Tub compared to controls. No effect was found in the first replicate ($t(20)=0.4595$, $p=0.6508$). Our hypothesis was not confirmed in the second α -tubulin PTMS considered, as the independent t-test to compare plasma levels of Tyr/Glu-Tot/Tub yielded no effect between groups in replicate 1 ($t(22)=0.1210$, $p=0.9048$), in replicate 2 ($t(19)=0.07172$, $p=0.9436$), and in the average of the replicates ($t(23)=0.2608$, $p=0.7966$).

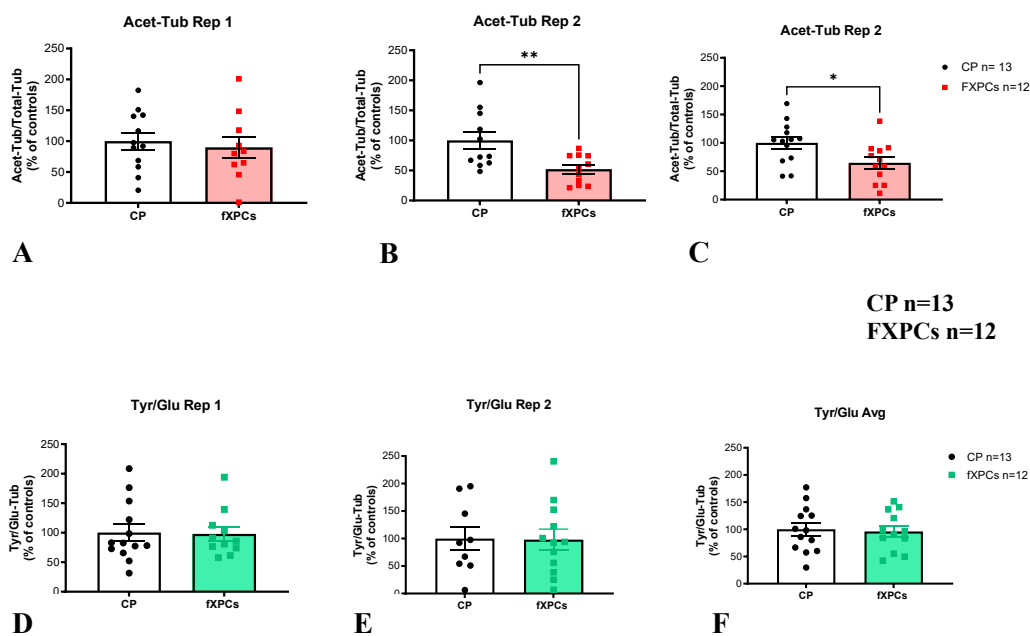


Figure 35: Comparison of Acet/Tub-Tot/Tub ratio and Tyr/Glu-Tot/Tub ratio between FXPCs and CP.

Two technical replicates were done for analysis of Acet/Tub-Tot/Tub ratio and for Tyr/Glu-Tot/Tub ratio. (A) No significant difference between groups was found in the first replicate. (B) A significant difference was found in the second replicate between groups. (C) A significant difference between groups was found when the replicates were averaged. (D) No significant difference between groups was found in the first replicate. (E) No significant difference was found in the second replicate between groups. (F) No significant difference is found between groups when the replicates were averaged. Data expressed as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$.

Electrochemiluminescent Elisa: Neurofilament light (NfL)

We finally hypothesised potential differences in NfL levels, however this hypothesis was not confirmed as the independent t-test revealed no significant difference in plasma levels of Neurofilament light chain (NfL) between groups ($t(27) = 0.5861$, $p = 0.5627$) (Figure 36).

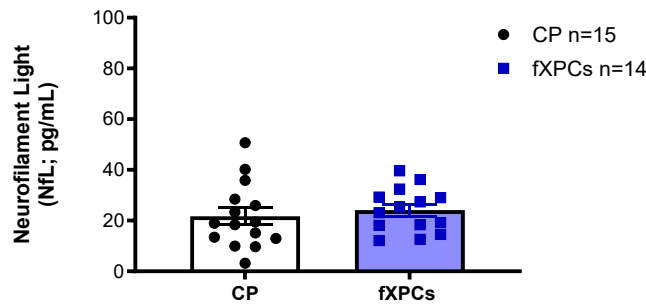


Figure 36: Comparison of neurofilament light (NfL) levels between CP and FXPCs. No significant difference was found in the analysis of NfL in plasma samples of individuals with FXPCs and CP. Data expressed as mean $\pm$ SEM.

Exploratory analysis

Our main goal with this exploratory analysis was to identify potential factors influencing the results. We first compared participants under medications and unmedicated participants. A one-way ANOVA was performed to compare Acet/Tub-Total/Tub ratio between participants under medications and unmedicated participants in the CP and in the FXPCs group revealing a significant group effect ($F(3, 21) = 3.839$, $p = 0.0246$). Particularly, the post hoc pairwise comparison showed no significant difference between the medicated and non-medicated participants in the CP group ($p = 0.2903$, Fisher LSD test), no significant difference between medicated and unmedicated participants ($p = 0.0667$), a significant difference between unmedicated CP and FXPCs ($p = 0.0034$), and a non-significant difference between medicated CP and FXPCs ($p = 0.5658$). In Tyr/Glu-Total/Tub no effect of medications was found between groups. A one-way ANOVA was performed to compare Tyr/Glu-Total/Tub ratio between participants under medication and unmedicated participants in the CP and FXPCs group producing no group effect ($F(3, 20) = 0.7855$, $p = 0.5160$). The post hoc pairwise comparison revealed no significant effect between medicated and unmedicated participants in the CP group ($p = 0.9534$), nor in the FXPCs group ($p = 0.1710$). No difference was shown between medicated CP and

FXPCs ($p=0.3720$) and non-medicated CP and FXPCs ($p=0.3418$) either. A one-way ANOVA performed to compare NfL levels between medicated and unmedicated participants produced no group effect [$F(3, 25) = 0.2812$, $p=0.2812$]. Specifically, the post-hoc pairwise comparison showed that no effect was there between medicated and unmedicated CP ($p=0.5191$) and FXPCs ($p=0.7649$), and between medicated CP and FXPCs ($p=0.4653$), and unmedicated CP and FXPCs ($p=0.7345$) (Figure 37).

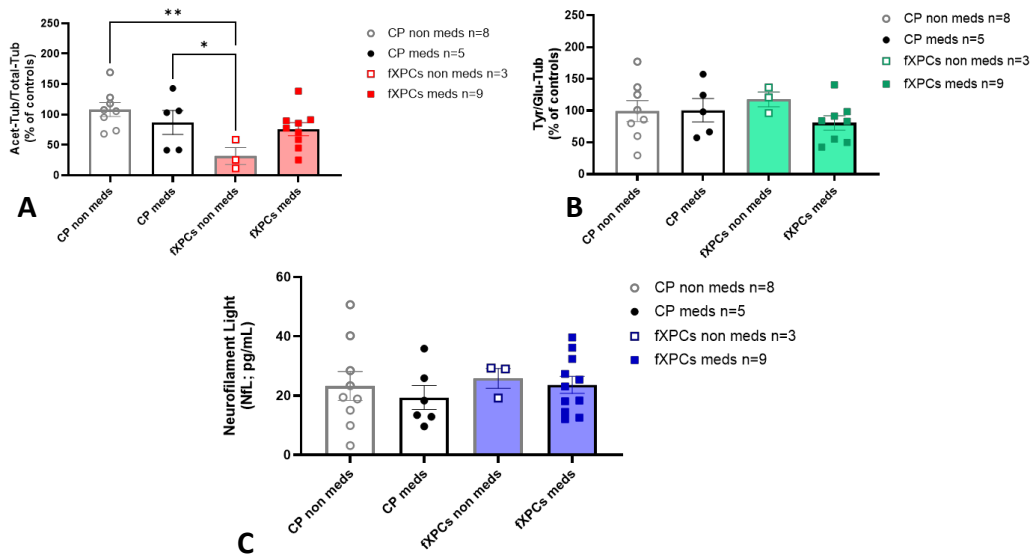


Figure 37: Comparison of α -tubulin PTMS and NfL between medicated and unmedicated patients in FXPCs and CP.

(A) Medicated (meds) and unmedicated (non-meds) participants were compared in both groups with Acet/Tub-Tot/Tub ratio. No effect of medication is shown in the CP group compared to the FXPCs unmedicated group. (B) Medicated (meds) and unmedicated (non-meds) participants were compared in both groups with Tyr/Glu-Tot/Tub ratio. No effect of medication was identified in the Tyr/Glu-Tot/Tub ratio when the groups were compared separated by medicated and unmedicated participants. (C) Medicated (meds) and unmedicated (non-meds) participants were compared in both groups with NfL levels. No effect of medication was shown in the NfL levels when groups were compared separated by medicated and unmedicated participants.

Data expressed as mean $\pm$ SEM * $p < 0.05$, ** $p < 0.01$

We additionally performed exploratory analysis to investigate the effects of certain variables such as medication use, age, CGG repeats and medical history. We only found one significant correlation in the CP group that revealed a relationship between medical history in the CP group, and Acet/Tub-Tot/Tub ratio ($r(10) = -0.6504$, $p = 0.0152$) (Figure 38) suggesting that a decrease in Acet/Tub-Tot/Tub ratio corresponds to a higher number of neurological conditions.

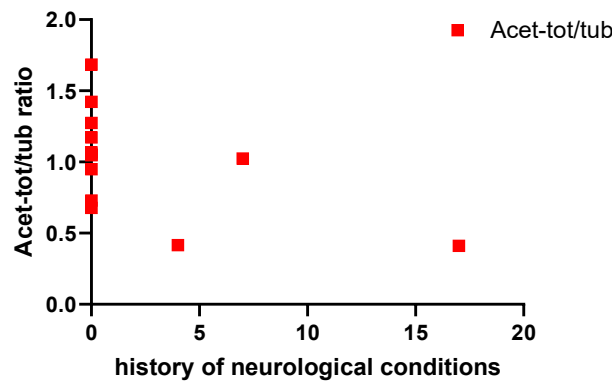


Figure 38: Correlation between medical history and Acet-tot/tub ratio in the CP group.

The figure shows a negative correlation between Acet-tot/tub ratio and history of neurological conditions, indicating an inverse relationship where to a higher number of conditions corresponds decreased levels of Acet-tot/tub ratio. "0" indicates no history of neurological conditions, while "1" corresponds to a history of panic attacks, "2" to depression, "3" to FXTAS, and "4" to a combination of depression and anxiety. Attention-Deficit/Hyperactivity Disorder (ADHD) and depression (5), ADHD with anxiety (6), autism (7), ADHD with anxiety and depression (8), migraines (9), anxiety with depression and migraine (10), migraine with anxiety (11), depression with developmental learning difficulties (12), migraines with FXTAS (13), Transient Ischemic Attack (TIA) (14), migraine with anxiety and ADHD (15), trigeminal neuralgia (16), epilepsy with migraines (17), depression with Post-Traumatic Stress Disorder (PTSD) and anxiety (18), Premenstrual Dysphoric Disorder (PMDD) (18), concussion (19), and migraine with depression (20).

This negative correlation between medical history of neurological condition and Acet-tot/tub ratio in the CP group was significant, indicating an inverse monotonic relation between the two factors: to lower levels of Acet-tot/tub ratio correspond different neurological or neuropsychiatric conditions. To zero neurological or neuropsychiatric conditions, corresponds higher levels of Acet-tot/tub ratio. However, no other significant correlations were present in the CP group between medical history and NfL ($r(10) = 0.2365$, $p = 0.4697$), or the Tyr/Glu-Total/Tub ratio ($r(10) = 0.04838$, $p = 0.8788$). Pearson correlation analysis in the FXPCs group did not reveal any significant relationship between age and the molecular measures (NfL, Acet/Tub-Total/Tub ratio, Tyr/Glu-Total/Tub ratio) did not: NfL ($r(12) = 0.04931$, $p = 0.8671$), Acet/Tub-Total/Tub ratio ($r(10) = 0.1526$, $p = 0.6359$), Tyr/Glu-Total/Tub ratio ($r(10) = -0.4377$, $p = 0.1547$). For the CP group we performed Spearman Rank correlation as at least one of the variables (NfL) was not normally distributed. These correlations did not reveal any significant relationship between age and the molecular measures (NfL, Acet/Tub-Total/Tub ratio, Tyr/Glu-Total/Tub ratio): did not NfL ($r(14) =$

0.1934, $p = 0.4702$), Acet/Tub-Total/Tub ratio ($r(10) = -0.4620$, $p = 0.1136$), Tyr/Glu-Total/Tub ratio ($r(10) = 0.2019$, $p = 0.5055$). Pearson correlation in the FXPCs group did not reveal any significant relationship between CGG repeat size and the molecular measures (NfL, Acet/Tub-Total/Tub ratio, Tyr/Glu-Total/Tub ratio): did not NfL ($r(5) = 0.6096$, $p = 0.1462$), Acet/Tub-Total/Tub ratio ($r(3) = -0.4741$, $p = 0.4198$), Tyr/Glu-Total/Tub ratio ($r(3) = -0.2012$, $p = 0.7455$). Unfortunately, only a handful of in-person participants provided information about the CGG repeat size, therefore final conclusions about these correlations cannot be drawn. The Spearman Rank correlation in the FXPCs group did not yield any significant correlation with medical history of neurological conditions: NfL ($r(12) = 0.3034$, $p = 0.2985$), Acet/Tub-Total/Tub ratio ($r(10) = 0.3062$, $p = 0.5000$), Tyr/Glu-Total/Tub ratio ($r(10) = 0.4804$, $p = 0.1667$).

3.3 Molecular experiment 2: Dysregulation of microtubule dynamics in FXS

3.3.1 Background

As introduced early this Chapter, we run an exploratory retrospective experiment on plasma samples of individuals with FXS to verify whether differences in plasma-based proteins alterations were present even between FXS and CP. As mentioned in 1.2.2, FXS is caused by a CGG repeat expansion on the *FMRI* gene that goes above <200 repeats. This excess of repeat size silences the gene and causes loss of FMRP protein. FMRP is important for many processes in neurodevelopment and for synaptic plasticity, and although many functions are yet to be discovered we know that it is an mRNA-binding protein and interacts with MAP1B, a microtubule-associated protein [263]. MAP1B is known for its regulator role of MT dynamics through phosphorylation. MAP1B activity involves several other proteins, and particularly GSK-3 is considered to mediate phosphorylation of MAP1B [265]. As already mentioned in 3.1.1, although many of the functions of the FMRP are yet to be uncovered, it is known that one of the effects is its impact on the GSK-3 signalling pathway. GSK-3 β is known to be involved in microtubule dynamics, and this protein together with FMRP interacts with microtubule associated protein 1b (MAP 1B). Inactivation of GSK-3 β is required to promote neurite growth and because loss of FMRP is associated with increased levels of GSK-3 β activation, investigating

microtubule dynamics could be of importance in FXS as well [270]. It has been shown that reducing FMRP levels reduces inhibitory phosphorylation of GSK-3 [264], and trying to restore this mechanism has been an important target for therapeutic interventions in FXS for example using lithium citrate in-vivo models to increase phosphorylation [264], to reduce hyperlocomotion [266], and to improve behavioural deficits in mice [267]. When tested in clinical trials with individuals with FXS some positive effects have been shown on symptoms [268]. Several GSK-3 inhibitors have been explored in recent years with promising results on in-vivo models, on cognition, protecting against seizures and improving behavioural phenotypes[272-274]; particularly GSK-3 β , which has a regulatory role in Tau mechanisms and interacts with microtubule associated proteins as well as FMRP [263, 269-271].

The role of MT dynamics in neurodevelopment

In the previous paragraphs we discussed the role of Microtubule Dynamics (MT) in neurodegeneration mechanisms, here we focus on its important role in brain development and defects could affect neurodevelopment. The MT cytoskeletal network plays a fundamental role during neuronal development, particularly during neurite formation, axon specification and dendrite branching. Additionally, the newly formed neurons depend on the dynamic MT for many active functions such as proliferation and migration, as well as in the extension of axons and dendrites, a fundamental step to enhance synaptic connections [192]. Neurodevelopmental disorders are characterised by cortical abnormalities and at the basis of these abnormalities the processes affected are usually the ones mentioned above, proliferation, migration, and differentiation. Neurodevelopmental disorders are therefore characterised by impairment in synaptic connectivity, and because of the important role played by MT in these processes it is clear that dysfunctions in MT can be linked to several neurodevelopmental disorders [304]. As seen above, tubulin post-translational modifications play a fundamental role in MT dynamics regulation (PTMS), and alteration of PTMs have been linked to several neurodegenerative disorders. Because of the role played in neurodevelopment by MT dynamics, dysregulation of PTMs can also be implied in neurodevelopmental disorders. Dysregulation of acetylation and tyrosination particularly, can be connected to abnormal neuronal development. It has been shown that tyrosination and acetylation regulate

neuronal migration of cortical plate neurons, and several other processes such as neuronal outgrowth, polarisation and synaptic connections, therefore dysregulation of tyrosination and acetylation can lead to disruption to all these fundamental processes [305]. Here we examine potential PTMs dysregulation in FXS patients as a potential marker of MT alterations. Additionally, we will pair this with analysis of NfL which has already been found overexpressed in CDD as shown in 1.5 in Figure 2 (unpublished data), potentially indicating axonal damage as discussed in 1.5.

3.3.2 Materials and Methods

Population

Plasma samples from individuals recruited in Argentina were received for analysis in Trinity College Dublin. The study was approved by the Ethical board of the Neurology Department of FLENI Hospital, in Buenos Aires Argentina [306] and then consent for analysis of samples was obtained by the School of Psychology Ethical Committee in Trinity College Dublin. Participants' legal guardian gave written consent for participating in the study. Table 24 outlines demographic information of participants.

	Control Participants (CP)	Fragile X Syndrome (FXS)
n	23	24
Age		
Children (CH) 4-12	11	11
Adults (AD)	12	13
Gender		
Male	20	21
Female	3	3
Medicated		
Yes	0	13
No	23	11

Table 24: Demographic information of the FXS population.

This table illustrates participants demographic characteristics such as age, gender and medications used for both groups, CP and FXPCs, which were gender and age matched.

Analysis of α -tubulin PTMs.

Analysis of α -tubulin PTMs through infrared Western blot assay was done by the PhD candidate. Plasma total protein concentration was analysed by Bradford Assay using BSA standards and uniformly equalised to 10 mg/ μ l, respectively. Samples were then diluted 2x in Laemmli buffer to a concentration of 5 mg/ μ l

before analysis by infrared Western blot for acetylated vs total α -tubulin, and tyrosinated vs de-tyrosinated (Glu) α -tubulin quantification. All Western Blot's were repeated 2 times for an n of 2 technical replicates following procedure described in 0. Microtubule dynamics were assessed by measuring the levels of three different α -tubulin PTMs – acetylated (Acet), tyrosinated (Tyr), and de-tyrosinated (Glu) α -tubulin. Acet was expressed as a ratio of total α -tubulin and Tyr was expressed as a ratio of Glu α -tubulin. These ratios are reflective of microtubule stability or dynamics, with a high Acet/Total α -tubulin ratio and low Tyr/Glu ratio being associated with reduced microtubule dynamics. All data was normalised to the average ratios of the control group for statistical analysis.

Electrochemiluminescent Elisa: NfL

Electrochemiluminescent Elisa was performed as described in 3.2.2.

Statistical analysis

Statistical comparisons were made between groups to answer our third research question (c) Is it possible to identify potential alterations in plasma-based proteins in FXPCs and in FXS? Are these alterations similar?) and verify whether differences are detectable in plasma-based proteins between FXS and CP. We used unpaired t-tests for comparing the two groups. Furthermore, we performed an additional exploratory analysis to verify the difference between medicated and unmedicated patients using one-way ANOVAs followed by Fisher's LSD pairwise comparisons with $p < 0.05$ set as the level for significance. All statistical tests were performed and graphed using Grahphad Prism v. 10.2.3.

3.3.3 Results

Western Blot analysis

We expected that plasma-based proteins would be altered in samples of individuals with FXS compared to a CP group, and we found microtubules showed dysregulation in the plasma of individuals with FXS when compared to plasma from age and gender-matched healthy controls, but this time in the Tyr/Glu α -tubulin, as opposed to the FXPCs group (Figure 39). While the independent t-test to compare plasma levels of Acet/Tub-Tot/Tub between CP and FXS did not produce any significant effect between groups putting together

children and adults ($t(42) = 0.1048$, $p = 0.9170$) nor when separating the groups (children ($t(17) = 0.5247$, $p = 0.6066$) and adults ($t(20) = 0.075$, $p = 0.5504$), the independent t-test to compare plasma levels of Tyr/Glu-Tot/Tub yielded a group effect in the analysis of children and adults combined ($t(39) = 2.628$, $p = 0.0122$), in the adults ($t(20) = 2.235$, $p = 0.0370$) but not in the children group ($t(17) = 1.386$, $p = 0.1835$).

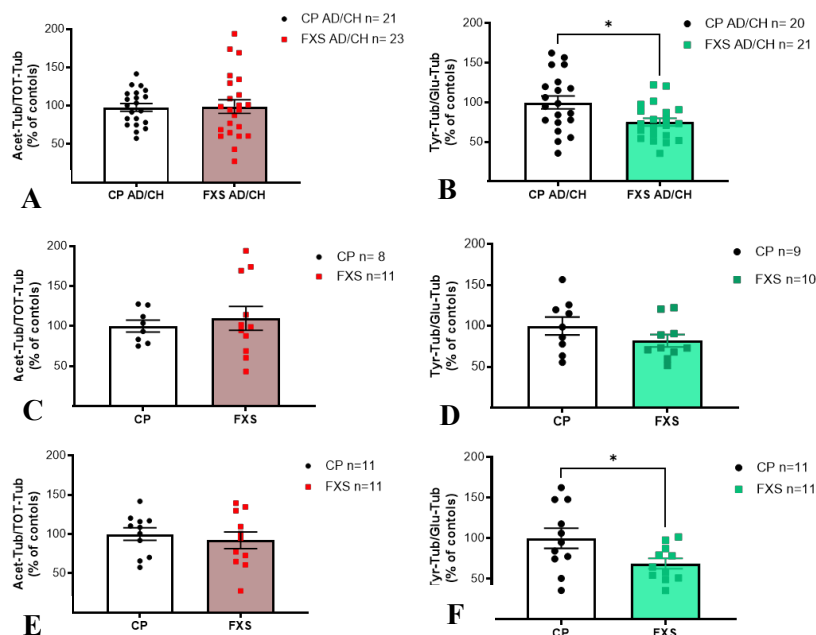


Figure 39: Comparison of Acet/Tub-Tot/Tub ratio and Tyr/Glu-Tot/Tub ratio between FXS and CP.

A/B: Analysis of α -tubulin PTMs in the plasma of individuals with FXS. (A) There were no significant differences in the Acet/Total α -tubulin ratio when comparing individuals with FXS with matched controls when analysing children (CH) and adults (AD) combined. (B) There was a significant difference in Tyr/Glu α -tubulin, that was decreased in FXS versus matched controls. **C/D Analysis of α -tubulin PTMs in the plasma of individuals with FXS, children (aged 4-12).** (C) There were no significant differences in the Acet/Total α -tubulin ratio when comparing children with FXS with matched healthy controls. (D) There was no significant difference in Tyr/Glu α -tubulin ratio. **E/F: Analysis of α -tubulin PTMs in the plasma of individuals with FXS, adults (aged 18-46).** (E) There were no significant differences in the Acet/Total α -tubulin ratio when comparing adults with FXS with matched healthy controls. (F) There was a significantly lower Tyr/Glu α -tubulin ratio in individuals with FXS compared to matched healthy controls in adults. * $p < 0.05$ versus matched healthy controls. Data expressed as mean $\pm$ SEM.

Electrochemiluminescent Elisa: Neurofilament light (NfL)

Furthermore, we expected to find differences in NfL levels to confirm our hypothesis of alterations in plasma-based proteins in FXS individuals compared to CP. However, our hypothesis was not confirmed as the independent t-test to

compare plasma levels of Neurofilament light chain (NfL) did not show any effect between groups [$t(45) = 0.2782$, $p = 0.7821$], potentially indicating no axonal damage in FXS individuals (Figure 40).

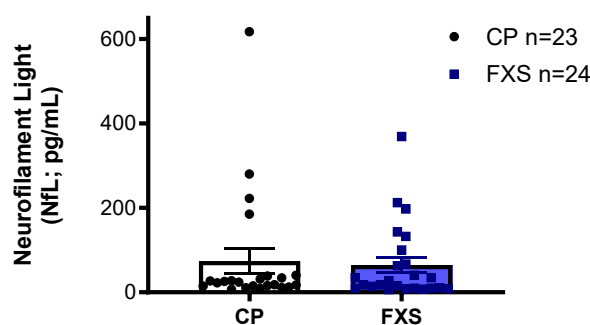


Figure 40: Comparison of neurofilament light (NfL) levels between CP and FXPCs. No significant difference was found in the analysis of NfL in plasma samples of individuals with FXS and CP. Data expressed as mean.

Exploratory analysis

Exploratory analysis to investigate the effects of medication did not reveal any significant influence. The one-way ANOVA to compare the CP group and the medicated and unmedicated participants in the Acet/Tub-Tot/Tub condition did not yield any group ($F(2, 38) = 0.3025$, $p = 0.7407$) showing that medications did not affect the results, while the one-way ANOVA to compare the CP group with medicated and unmedicated participants in the Tyr/Glu-Tot/Tub showed a group effect ($F(2, 38) = 3.372$, $p = 0.0449$). The post-hoc analysis of the Tyr/Glu-Tot/Tub revealed that a significant difference is present between CP and medicated FXS participants ($p = 0.0367$ Fisher LSD test) and between CP and unmedicated FXS ($p = 0.0432$), but not between medicated and unmedicated FXS ($p = 0.9033$), indicating again that medications did not affect the results.

As seen in 3.3.3, both increase in Acetylated tubulin and decrease in Tyrosinated over detyrosinated tubulin are associated with hyper stabilised microtubules, which results in a dysregulated dynamics having an impact on all the important functions that MT dynamics carry out for neurons and synaptic plasticity. In the data analysed no significant difference was found in Acet/Total α -tubulin compared to healthy controls in either of the age groups. Additionally, no effect of medications was found on acetylation. However, the Tyr/Glu α -tubulin ratio was significantly decreased in individuals with FXS compared to healthy controls when combining both age groups. The same significant difference was

found in the adult group no significant difference was found in the children group. Medications did not modify the results of the Tyr/Glu α -tubulin ratio (Figure 41).

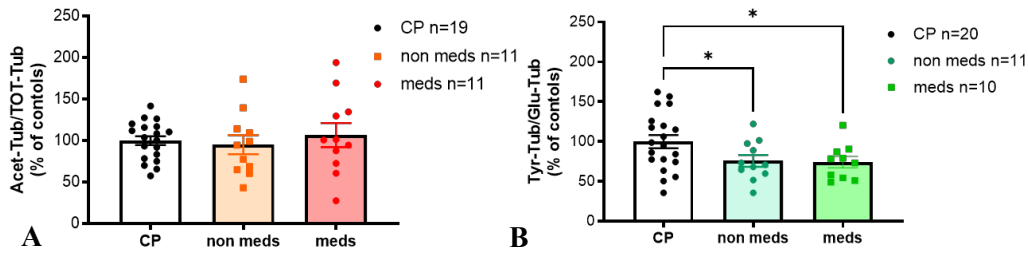


Figure 41: Comparison of α -tubulin PTMs and NfL in the plasma of medicated (meds) and unmedicated (non-meds) FXS individuals.

(A) There was no effect of medication on the Acet/Total α -tubulin ratio (all ages combined). (B) There was no effect of medication on the Tyr/Glu α -tubulin ratio in FXS (all ages combined) versus matched healthy controls. Data expressed as mean $\pm$ SEM. * $p < 0.05$

3.4 Combining molecular and linguistic measures in FXPCs

3.4.1 Background

In this paragraph we try to answer our fourth research question: Is it possible to find links between linguistic and molecular measures by combining molecular and linguistic measures to advance development of treatments for FXPCs? One of our goals here, is to investigate potential specific measures that combined could function as complex indications of differentiation between a control group and a clinical population. Therefore, here we will combine the molecular measures with the linguistic ones, hypothesising that the molecular measures were connected with the language assessments. The rationale for this can be found in the complex neurobiology of language. We have seen in Chapter 1 how complex is the language neural network, involving several different brain areas and pathways, from frontotemporal to posterior temporal cortical areas, connected together through white matter fibre bundles such as the arcuate fascicle and the superior longitudinal fascicle for example [307]. White matter pathways have been shown to be fundamental in memory and learning, particularly for experience-dependent plasticity [308], and because of the importance of MT dynamics in several axonal mechanisms and in plasticity, alterations in these mechanisms could be reflected in tasks dependent on memory and learning such as linguistic abilities. Additionally, because NfL can reflect

axonal damages and it has already been identified as a marker of neurodegeneration, we believe it could potentially be combined with behavioural measures, including language abilities, that can be altered at early stages of cognitive decline [309]. Furthermore, uncovering the basis of the neurobiology of language has gained attention in last decade, because of the discovery of certain genes connected to language impairment like *FOXP2*, or other regulatory molecular mechanisms such as for example Micro RNAs like Mir-137, a target of *FOXP2*, which has been implicated in the pathogenesis of several disorders where language is affected including autism, and schizophrenia [308]. This opened the field of connecting molecular neuroscience to the language science, and led to the discovery of several other associations, like the connections between *RBFOX2* which has been associated with language measures [310]. If our hypothesis is correct, the two measures combined could potentially function as a complex measure to be further investigated in the future for potential differential diagnosis and for therapeutic targets.

3.4.2 Materials and methods

Population

We combined analysis of molecular measures and linguistic measures for the in-person FXPCs participants who entered the study, because they provided both a blood sample as well as participating in the linguistic assessment. Demographic details of the population can be found in section 3.2.2 of this chapter.

Data processing and Statistical Analysis

To verify our hypothesis that the molecular measures could be factors correlating with the language outcomes, multiple linear regression models were conducted to examine the relationships between molecular measures, treated as independent variables, and language outcome measures. Independent variables included NfL, Acet-tot/tub, Tyr-Glu/tub, and genotype, which was dummy coded as FXPCs (1) or CP (2). For Acet-tot/tub and Tyr-Glu/tub the raw values of the ratios were used for comparison with the linguistic values. Four distinct models were developed to evaluate the impact of these variables on the dependent variables, which comprised language outcomes: RTs for objects and actions in picture naming and RTs for objects and actions in the VWP paradigm. To verify correlation between the two types of measures in accuracy for objects

and actions in picture naming was correlated with NfL, Acet-tot/tub, Tyr-Glu/tub, and genotype, which was dummy coded as FXPCs (1) or CP (2) through a series of Spearman rank correlations as some of the variables were not normally distributed. In the picture description task, only measures that demonstrated a clear group effect between CP and FXPCs, indicating significant differences, were included. This subset consisted of block, word revisions, phrase revisions, Total SLD, percentage of adjectives, and retracing numbers. However, as these variables were nonparametric and did not meet normal distribution criteria, log transformation was attempted to fit them within a linear regression model. Log transformation is applied to skewed or non-normally distributed data to meet the assumptions of statistical methods, such as linear regression, by computing the logarithm of each data point [311]. Because many outcome measures were near or equal to zero (indicating, for instance, a lack of disfluency in participants), a constant value of 1 was added to each data point before applying the log transformation to prevent undefined values [312]. Despite this adjustment, the log transformation did not produce a normal distribution. Consequently, Spearman rank correlation was employed to assess the direction of these relationships with the independent variables. P-values corrected for multiple comparison with Benjamini–Hochberg False Discovery Rate (FDR), a test that controls the proportion of false positive and represent the perfect balance between reducing this possibility and the potential for false negatives as explained in section 2.3.4. IBM SPSS Statistics version 29.0.1.0 (171) was used for log transformation, while GraphPad Prism 10.2.3 (403) was used for the linear regression models.

3.4.3 Results

To answer our final research question (d) Is it possible to find links between linguistic and molecular measures by combining molecular and linguistic measures to advance development of treatments for FXPCs?), we hypothesised that the molecular measures would be factors influencing the linguistic outcome in RTs, and that the two types of measures would be correlated in accuracy. However, the first part of our hypothesis was not confirmed here none of the regression model with RTs was significant in the picture naming or in the visual word paradigm task, and no predictors were identified.

Picture naming task

In the Picture naming task, none of the models was significant. The multiple regression between molecular measures and RTs in object was not statistically significant, $F(4,20) = 0.2707$, $p = 0.8934$, indicating that the predictors collectively do not explain a significant portion of the variance in response times. The total sum of squares (SS) was 610083, with 31331 attributed to the regression model and 578752 to the residual variance. Additionally, the model's R-squared value was 0.05136, suggesting that only about 5.1% of the variance in response times is explained by the predictors, indicating a poor goodness-of-fit. The residual sum of squares (SS) was 578752, with a mean square error of 28938, and the degrees of freedom were 20. These results, along with the low R-squared, indicate that a large portion of variance remains unexplained by the current predictors. In summary, this regression analysis did not identify any significant predictors for response times in object naming as shown in Table 25. The non-significant findings across all predictors, along with the low goodness-of-fit, suggest that additional factors, beyond those included in this analysis, may influence response times in objects naming.

Model					
Analysis of Variance	SS	DF	MS	F (DFn, DFd)	P value
Regression	31331	4	7833	$F(4, 20) = 0.2707$	$P = 0.8934$
NfL	27349	1	27349	$F(1, 20) = 0.9451$	$P = 0.3426$
FXPCs (1) CP (2)	7.004	1	7.004	$F(1, 20) = 0.0002420$	$P = 0.9877$
Acet-tot/tub	1096	1	1096	$F(1, 20) = 0.03788$	$P = 0.8476$
Tyr-Glu/tub	4613	1	4613	$F(1, 20) = 0.1594$	$P = 0.6939$
Residual	578752	20	28938		
Total	610083	24			

Table 25: Multiple linear regression between Objects RTs in the picture naming task and molecular measures.

The ANOVA table shows multiple linear regression analysis for the dependent variable "Object RTs" in the Picture naming task. The model does not significantly explain variability in RTs for object naming.

The second multiple regressions between molecular measures and RTs in action did not achieve statistical significance either (Table 26), $F(4, 20) = 0.3791$, $P = 0.8209$, indicating that the predictors do not collectively explain variance in the outcome. The total sum of squares (SS) was 641824, with 45232 attributed to the regression model and 596592 to the residual variance. Additionally, the model's R-squared value was 0.07047, suggesting that only about 7% of the

variance in response times is explained by the predictors, indicating a poor goodness-of-fit. The residual sum of squares (SS) was 596592, with a mean square error of 29830, and the degrees of freedom were 20. These results, along with the low R-squared (0.07047), indicate that a large portion of variance remains unexplained by the current predictors. The non-significance of all predictors suggests that molecular markers NfL, genotype, Acet-tot/tub, and Tyr-Glu/tub do not substantially influence the response times for action naming.

Model					
Analysis of Variance	SS	DF	MS	F (DFn, DFd)	P value
Regression	45232	4	11308	F (4, 20) = 0.3791	P=0.8209
NfL	8557	1	8557	F (1, 20) = 0.2869	P=0.5981
FXPCs(1)CP(2)	9340	1	9340	F (1, 20) = 0.3131	P=0.5820
Acet-tot/tub	14205	1	14205	F (1, 20) = 0.4762	P=0.4981
Tyr-Glu/tub	685.4	1	685.4	F (1, 20) = 0.02298	P=0.8810
Residual	596592	20	29830		
Total	641824	24			

Table 26: Multiple linear regression between Actions RTs in the picture naming task and molecular measures.

The ANOVA table shows multiple linear regression analysis for the dependent variable “Actions RTs” in the Picture naming task. The model does not significantly explain variability in RTs for action naming.

Visual word paradigm

In the visual word paradigm, the regression model between molecular measures and RTs for the object condition did not achieve statistical significance, $F(4, 19) = 2.252$, $P = 0.1016$, suggesting that the predictors collectively explain about 32.16% of the variance ($R^2 = 0.3216$). Only one predictor, genotype (FXPCs vs CP) achieved statistical significance, significance $F(1, 19) = 5.770$, $P = 0.0267$ thus the different groups contributed to the model. Because the coefficient is negative -109.3, the relationship is inverse indicating potentially that the FXPCs group (dummy coded as 1) is associated with higher values in the VWP outcome compared to the CP group (dummy coded as 2). However, this difference was expected as it confirms our findings of a difference between CP and FXPCs discussed in 2.4. The total sum of squares (SS) was 244392, with 78605 attributed to the regression model and 165788 to the residual variance, indicating that a considerable amount of variance remains unexplained by this model. These findings show genotype as the only potential meaningful predictor to the outcome variable as shown in Table 27. However, a considerable amount of variance remains unexplained in this model, potentially pointing to other influential factors.

Model					
Analysis of Variance	SS	DF	MS	F (DFn, DFd)	P value
Regression	78605	4	19651	F (4, 19) = 2.252	P=0.1016
NfL	0.03947	1	0.03947	F (1, 19) = 4.523e-006	P=0.9983
FXPCs(1) CP(2)	50348	1	50348	F (1, 19) = 5.770	P=0.0267
Acet-tot/tub	4.071	1	4.071	F (1, 19) = 0.0004666	P=0.9830
Tyr-Glu/tub	4503	1	4503	F (1, 19) = 0.5160	P=0.4813
Residual	165788	19	8726		
Total	244392	23			

Table 27: Multiple linear regression between Objects RTs in the VWP and molecular measures.

The ANOVA table shows multiple linear regression analysis for the dependent variable “Object RTs” in the Visual Word Paradigm task. The model does not significantly explain variability in RTs for object naming; however, genotype is the only significant predictor indicating FXPCs associated with higher RTs compared to CP.

The second regression model in the visual word paradigm between molecular factors and RTs in the action condition was not statistically significant either, $F(4,19)=1.138$, $P=0.3687$ suggesting once again that the predictors do not explain a meaningful amount of variance ($R\text{-squared} = 0.1933$), indicating that only about 19.33% of variance is explained by the model. The total sum of squares (SS) was 577,059, with 111,521 attributed to the regression model and 465,538 to residual variance. Once again, this model shows that none of these molecular measures are significant predictors of the VWP actions as shown in Table 28.

Model					
Analysis of Variance	SS	DF	MS	F (DFn, DFd)	P value
Regression	111521	4	27880	F (4, 19) = 1.138	P=0.3687
NfL	30161	1	30161	F (1, 19) = 1.231	P=0.2811
FXPCs(1) CP(2)	100.9	1	100.9	F (1, 19) = 0.004120	P=0.9495
Acet-tot/tub	14934	1	14934	F (1, 19) = 0.6095	P=0.4446
Tyr-Glu/tub	91256	1	91256	F (1, 19) = 3.724	P=0.0687
Residual	465538	19	24502		
Total	577059	23			

Table 28: Multiple linear regression between Actions RTs in the VWP and molecular measures.

The ANOVA table shows multiple linear regression analysis for the dependent variable “Action RTs” in the VWP. The model does not significantly explain variability in RTs for action naming; however, a non-significant trend shows Tyr-Glu/tub ratio as a potential predictor.

Correlation between accuracy and disfluencies, and molecular measures.

When we correlated accuracy and molecular measures our hypothesis was confirmed as we found a few correlations between molecular measures and linguistic measures.

Picture naming task

A series of Spearman rank correlations were performed here as some of the variables were not normally distributed and some correlations were found confirming our hypothesis of potential connections between the molecular and linguistic analysis. In the FXPCs group a moderate to strong correlation was found between Tyr-Glu/tub ratio and accuracy with errors and dysfluencies in the object condition ($r(12) = 0.6643$, $p = 0.0219$, $q = 0.0108$). This potentially indicates a monotonic relation where the Tyr-Glu/tub ratio increases as well as the percentage of errors and dysfluencies in the object condition. The correlation with errors and dysfluencies in the action condition was not significant ($r(12) = 0.3684$, $p = 0.2374$, $q = 0.4202$). No other correlations were found, between NfL and accuracy, with errors and dysfluencies in the object condition ($r(14) = 0.08361$, $p = 0.7761$, $q = 0.8714$), or errors and dysfluencies in the action condition ($r(14) = -0.1499$, $p = 0.6065$, $q = 0.7278$). Similarly, the correlation analysis between Acet-tot/tub ratio and accuracy in the picture naming task did not reveal any significant correlations with errors and dysfluencies in the object condition ($r(12) = 0.05604$, $p = 0.8640$, $q = 0.8714$), or errors and dysfluencies in the action condition ($r(12) = 0.3533$, $p = 0.2571$, $q = 0.4202$). In the CP group, only one correlation was found between Acet-tot/tub ratio and accuracy in the picture naming with errors and dysfluencies in the object condition ($r(16) = 0.2801$, $p = 0.0235$), which however did not survive FDR correction ($q = 0.0705$), suggesting a potential correlation where the Acet-tot/tub ratio increases as well as the percentage of errors and dysfluencies in objects, but however this result was not significant. No significant correlation was found with errors and dysfluencies in the action condition ($r(16) = 0.3439$, $p = 0.1914$, $q = 0.4202$). The correlation between NfL and accuracy did not show any significant association either, with errors and dysfluencies in the object condition ($r(16) = 0.04418$, $p = 0.8714$, $q = 0.8714$), or errors and dysfluencies in the action condition ($r(16) = 0.2863$, $p = 0.2801$, $q = 0.4202$). Similarly, the correlation analysis between Tyr-Glu/tub ratio and accuracy did not reveal any significant correlations with errors

and dysfluencies in the object condition ($r(16) = 0.1561$, $p = 0.5611$, $q = 0.8714$), or with errors and dysfluencies in the action condition ($r(16) = -0.001476$, $p = 0.9980$, $q = 0.9980$).

Picture description task

Here again most of the correlations were not significant, however we found some interesting significant relationships that reinforced our hypothesis of a correlation between molecular and linguistic measures. The correlation between NfL and the picture description language measure in the FXPCs group did not reveal any significant relationship, with the block ($r(11) = 0.1130$, $p = 0.7148$, $q = 0.8631$), Wordrevisions ($r(11) = 0.2416$, $p = 0.4280$, $q = 0.8560$), Phraserevisions ($r(11) = 0.1115$, $p = 0.7249$, $q = 0.8631$), Total SLD ($r(11) = 0.05495$, $p = 0.8631$, $q = 0.8631$), percentage of adjectives ($r(11) = 0.5182$, $p = 0.0723$, $q = 0.4338$) and retracing number ($r(11) = 0.2620$, $p = 0.3840$, $q = 0.8560$) Table 29. Similarly the correlation with the CP group did not reveal any significant relationship with the block ($r(13) = 0.05528$, $p = 0.8459$, $q = 0.9999$), Wordrevisions ($r(13) = 0.1992$, $p = 0.5048$, $q = 0.9999$), Phraserevisions ($r(13) = -0.3093$, $p = 0.4000$, $q = 0.9999$), Total SLD ($r(13) = 0.03571$, $p = 0.9031$, $q = 0.9999$), percentage of adjectives ($r(13) = 0.08758$, $p = 0.7555$, $q = 0.9999$) and retracing number ($r(13) = 0.000$, $p = >0.9999$, $q = 0.9999$). The correlation between Acet-tot/tub ratio and the picture description language measure in the FXPCs group only revealed one negative correlation between Acet-tot/tub against percentage of adjectives, that however did not survive FDR correction ($r(9) = -0.6388$, $p = 0.0392$, $q = 0.2352$). No other significant correlations were found between Acet-tot/tub and the block ($r(9) = -0.01911$, $p = 0.9613$, $q = 0.9828$), wordrevisions ($r(9) = -0.01055$, $p = 0.9828$, $q = 0.9828$), Phraserevisions ($r(9) = -0.09460$, $p = 0.7636$, $q = 0.9828$), Total SLD ($r(9) = 0.1822$, $p = 0.5893$, $q = 0.9828$), and retracing number ($r(9) = -0.2361$, $p = 0.4784$, $q = 0.2352$). In the same way, no significant correlations were found between Acet-tot/tub ratio and the picture description language measure in the CP group. One correlation was present with the retracing number ($r(11) = -0.5855$, $p = 0.0490$, $q = 0.2307$), which however did not survive FDR correction. No other correlations were found with the block ($r(11) = -0.1074$, $p = 0.7307$, $q = 0.7307$), Wordrevisions ($r(11) = -0.4368$, $p = 0.1538$, $q = 0.2307$), Phraserevisions ($r(11) = -0.3086$, $p = 0.4615$, $q = 0.5538$), Total SLD ($r(11) = -$

0.5110, $p = 0.0776$, $q = 0.2307$), and percentage of adjectives ($r(11) = -0.4264$, $p = 0.1469$, $q = 0.2307$). Finally, the correlation between Tyr-Glu/tub ratio and the picture description language measure in the FXPCs group revealed one significant correlation between Tyr-Glu/tub ratio and Total SLD ($r(9) = 0.8455$, $p = 0.0018$, $q = 0.0108$), indicating a monotonic relation where the Total SLD number increases as well as the Tyr-Glu/tub ratio. No other significant correlations were found between Tyr-Glu/tub ratio and the block ($r(9) = 0.5244$, $p = 0.1019$, $q = 0.2038$), Wordrevisions ($r(9) = 0.06318$, $p = 0.8631$, $q = 0.8631$), Phraserevisions ($r(9) = 0.5394$, $p = 0.0909$, $q = 0.2038$), the percentage of adjectives ($r(9) = 0.2885$, $p = 0.3857$, $q = 0.4628$), and the retracing number ($r(9) = 0.3708$, $p = 0.2584$, $q = 0.3876$). In the CP group, no correlations were found with the block ($r(11) = -0.1342$, $p = 0.6656$, $q = 0.9734$), Wordrevisions ($r(11) = 0.4455$, $p = 0.1410$, $q = 0.4614$), Phraserevisions ($r(11) = -0.4629$, $p = 0.1538$, $q = 0.4614$), Total SLD ($r(9) = -0.005495$, $p = 0.9929$, $q = 0.9929$), the percentage of adjectives ($r(11) = -0.3549$, $p = 0.2329$, $q = 0.4658$), and the retracing number ($r(11) = 0.09759$, $p = 0.8112$, $q = 0.9734$).

These results indicate that some correlations are present, and that particularly the Tyr-Glu/tub ratio significantly correlate with the total percentage of disfluencies (Total SLDTD) in the picture description task, and with accuracy in the object condition of the picture naming (Table 29). These findings hold promise for combining molecular and linguistic measures confirming our hypothesis that they are connected. We will further explore this aspect in the discussion section 3.5.

CP	Acet-tot/tub	Tyr-Glu/tub	NfL
retracing number	* (p=0.0490, q= 0.2307) R = -0.5855	N/S	N/S
Accuracy picture naming object	*(p=0.0235, q=0.0705) R = 0.5685	N/S	N/S
FXPCs	Acet-tot/tub	Tyr-Glu/tub	NfL
Total SLDTD	N/S	** (p=0.0018, q= 0.0108) R=0.8455	N/S
Accuracy picture naming object	N/S	* (p=0.0219, q= 0.0108) R= 0.6643	N/S
Percentage of adjectives	* (p=0.0392, q= 0.2352) R=-0.6388	N/S	N/S

Table 29: Summary of findings in correlation between molecular and linguistic measures.

The table highlights the main findings when correlating linguistic and molecular measures. Most of the findings are not significant after FDR correction, however, the correlations with Tyr-Glu/tub remain significant after correction, potentially indicating a connection with this molecular measure.

3.5 Discussion

In this chapter we addressed our third research question with molecular experiment 1 and 2.

c) Is it possible to identify potential alterations in plasma-based proteins in FXPCs and in FXS? Are these alterations similar?

We expected to find difference between FXPCs/FXS and CP in both experiments. In the molecular experiment 1 we show that in a group of female carriers without FXTAS it has been possible to identify differences with controls at a molecular level. We found a decrease in Acetylated over Total α -tubulin in the FXPCs group compared to the healthy control group. This decrease in acetylation has been observed in a number of neurodegenerative disorders. In Alzheimer's Diseases a strong reduction in acetylated α -tubulin has been observed in neurons containing neurofibrillary tangles (NFT)[313] in postmortem brain tissue, in AD cells with patient mitochondrial DNA and AD brains a decrease in acetylation was correlated to mitochondrial dysfunction where SIRT2, a tubulin deacetylase regulating acetylation in microtubule, is overactivated influencing regulation of microtubules dynamics[314]. In Huntington's disease (HD) a reduction in acetylation was observed in HD brains

and in the same study Histone deacetylase (HDAC) inhibitors are able to increase acetylation restoring MT stability [315]. Even transgenic mice expressing two different mutations leading to Charcot-Marie-Tooth disease (CMT), the most common inherited peripheral nervous system disorder, showed a decrease in acetylated α -tubulin causing deficits in axonal transport, a decrease which was increased by the use of histone deacetylase 6 (HDAC6), which restored axonal transport damages and rescued the CMT phenotype in mice [316]. In another study on CMT type 2A (CMT2A) mouse model, decrease in acetylation was again restored by HDAC6, reinforcing the idea that MT dynamic dysregulation could be involved in the pathogenesis of the disorder and HDAC6 could be therapeutic target [317]. The same finding of reduction in acetylated α -tubulin was found in a study comparing plasma samples of individuals affected by CMT and healthy controls, run by Ulysses Neuroscience Ltd. (unpublished data), confirming that potentially expression of α -tubulin PTMs in plasma could be indicative of similar mechanisms happening in the brain. The same reduction in acetylated α -tubulin has been observed in amyotrophic lateral sclerosis (ALS), where again HDAC6 was shown to be a potential interesting therapeutic target [318]. Additionally, deficits in axonal transport have been found in a *Drosophila* model of Leucine-rich repeat kinase 2 (LRRK2) mutations, one of the most common genetic causes of Parkinson's Disease (PD), potentially related the importance of LRRK2 protein in many cellular processes and its interaction with microtubules. In this model microtubule acetylation was increased using deacetylase inhibitors, revealing a potential therapeutic mechanisms for Parkinson's disease (PD) as well [319]. In all of the above reduced acetylated α -tubulin was associated with deficits in axonal transport as acetylation is believed to be a regulator of this process in microtubules. Additionally, all the studies mentioned suggest the use of HDAC6 inhibitors to restore acetylation and axonal transport. The use of these inhibitors had the effect of improving symptoms and cognitive deficits as well, suggesting that acetylation of α -tubulin could also be involved in the pathogenesis of these diseases [286]. In this sample we are not considering participants affected by neurodegeneration, but some of the mechanisms that might be involved in the development of neurodegeneration in FXPCs, have been shown to be present in FXPCs without FXTAS [4]. We have seen that mitochondrial dysfunction is connected to α -tubulin acetylation in AD, and that acetylation is believed to play an important role in axonal transport.

FMRP is important in the brain for many cellular functions, including development in embryos and a regulatory role in synaptic plasticity throughout the lifespan. We have mentioned in 3.1.1 that FMRP interacts with MAP1B a known regulator of MT dynamics, whose phosphorylation is mediated by GSK-3, which is believed to have an important role in axonal growth and when FMRP is reduced it has been shown a reduction of inhibition of phosphorylation, potential influencing MT dynamics and axonal mechanisms. In individuals with by the full mutation of the *FMRI* gene, hence affected by FXS, the levels of FMRP are very low or the protein could be absent, in premutation carriers levels of FMRP are inversely correlated with the CGG repeat number; therefore premutation carriers with more than 120 CGG repeats can display significant low levels of FMRP [320]. However, most of the clinical symptoms experienced by FXPCs, are usually associated mostly with the RNA toxicity. The presence of upregulation of *FMRI* mRNA (directly correlated with the number of CGG repeats), triggers a process of RNA toxicity, where the excess *FMRI* mRNA prevents certain proteins important for neuronal mechanisms from carrying out their tasks, and this leads to the formation of inclusions in cells and tissues, including neurons, astrocytes, peripheral nervous system, pancreas, heart and others [320]. There is general agreement among researchers that both low levels of FMRP and RNA toxicity contributes to the clinical involvement of premutation carriers. Mitochondrial dysfunctions have also been observed in FXPCs with and without FXTAS, and deficits in mitochondria have been shown in some neurodevelopmental disorders and in neurodegenerative disorders such as AD and PD. Because mitochondrial dysfunction has been connected to α -tubulin acetylation in AD, similar mechanisms could be in place in FXPCs, although mitochondrial dysfunction in carriers has been primarily associated to deficits in transport of 3 proteins into the mitochondria, were also lower expression of mitochondria protein was observed to precede in age and CGG number the appearance of clinical symptoms of FXTAS [321]. Because of the deficits in mitochondrial transport and in general the lower levels of mitochondria found both in FXPCs and in FXTAS, and because HDAC6 inhibitors has been proven to improve axonal transport of mitochondria this could represent a therapeutical intervention. We believe that both potential reduction of FMRP and RNA toxicity could be involved in the dysregulation found in plasma samples of FXPCs, however we did not analyse FMRP levels

in the sample collected and the number of CGG repeat expansion was unavailable for most of the participants, therefore further analysis on a larger sample size would be required to confirm the results. We were expecting to find a correlation with the CGG repeat size and the results of the molecular analysis, but for the reasons stated above no relation was found. The exploratory correlation analysis in general did not show any relevant correlation between the demographic factors collected and the molecular analysis, except for a negative correlation between the previous medical history of neurological conditions and the Acet/Tub-Total/Tub ratio potentially suggesting a monotonic relation between the number of neurological conditions and decrease in Acet/Tub-Total/Tub ratio in the CP group. This could potentially indicate that certain neurological conditions could contribute to decrease the baseline of acetylation, which is higher when the number of neurological conditions is 0 and becomes lower when participants are affected by different neurological condition, such as for example the participants with the lowest Acet/Tub-Total/Tub ratio were affected by epilepsy and depression and anxiety. Nonetheless, the comparison between medicated and unmedicated participants showed a trend to significance with a potential increase in acetylation in medicated FXPCs. However, these findings are not conclusive as the result was not significant and potentially a larger sample size is needed to confirm these results. Whether these findings indicate the possibility of potentially identify prodromal signs of the risk of developing neurodegeneration, or whether they are simply indicative of alterations present in the body of individuals affected by the Fragile X Premutation it cannot be confirmed at this stage: however, the dysregulation found in acetylated α -tubulin could potentially indicate promising paths to be explored to develop new therapies. One limitation to be considered is the small sample size that does not allow us to draw final conclusions, and the variability present between the two replicates in the acetylated α -tubulin analysis. Unfortunately, due to lack of additional fundings it was not possible to repeat the analysis.

In the molecular experiment 2, we focused on FXS individuals to answer the second part of our third research question and investigate whether FXS participants also display an abnormal expression of plasma-based proteins compared to CP. We expected to find alterations compared to the CP group, and we demonstrate here that similar alterations are present in plasma samples of

individuals with FXS. We did not find any alteration in levels of Acet/Total, but we found a decrease in Tyr/Glu α -tubulin ratios in plasma samples of these patients. Surprisingly, we only found this pattern in adults and not in children, potentially indicating a progressive stabilisation in adulthood, a theory supported also by the higher variability found in the children group compared to adults. Molecular differences between FXS children and adults have been already found in literature [322], suggesting age related changes in this population. Unfortunately, we did not analyse samples from teenagers' individuals, an analysis that would help understanding the potential progression of the alterations identified. We believe that plasma samples provide an indirect measure of potential mechanisms happening in the brain, because similar patterns were found in mice, and in general increased MT stability is considered a characteristic of neurodevelopmental disorders, as reduced MT dynamics was found in several disorders such as Type I Lissencephaly, Polymicrogyria and in Autism Spectrum Disorders (ASD) [192], as well as schizophrenia, Down Syndrome and Epilepsy [323], and CDD as we have mentioned earlier [324]. The shift towards stabilisation in adults compared to children, potentially indicates an inclination towards stabilisation in adulthood, while in childhood a similar pattern is present between control participants and FXS individuals. Unfortunately, we did not have availability of samples from teenagers, but we believe that analysing that age range could complete a preliminary picture of potential Microtubule PTMs alterations, drawn with these results throughout the lifespan in FXS. The Ulysses neuroscience team also found similar alterations in hippocampus of *Fmr1* KO mice (3 months old), where the increase in the Acet/TOT and the decrease in the Tyr/Glu α -tubulin ratios was unsurprisingly more pronounced, given that the samples were taken from the brain (unpublished data). These findings support the possibility of using α -tubulin PTMS as potential biomarker in neurological disorders and therapeutic target in neurodevelopmental disorders, considering also that this is a peripheral fluid that do not require invasive and complicated procedure such as CSF collection [16]. Additionally, as mentioned in 1.3.1, studying rare diseases led to a collaborative competition among researchers to explore new therapeutic targets to tackle common issues that can be applied to more than one rare disease [16]. Particularly, α -tubulin PTMS have been successfully studied with promising results in CDD by Ulysses Neuroscience, where evidence of decrease in MT

dynamics was shown, particularly increased levels of Acet/Total and decreased levels of the Tyr/Glu α -tubulin ratios in plasma samples of patients with CDD and similar changes were found in hippocampi of CDKL5 knock out mice (manuscript under submission). Our similar findings in FXS patients, confirmed the importance of keep studying rare diseases and potential measures that could be applied across disorders.

As mentioned in 3.1.1, the importance of investigating α -tubulin PTMS is their potential to become potential therapeutic targets. To test this hypothesis the Ulysses Neuroscience team treated *Fmr1* KO mice with two GSK-3 β inhibitors. Interestingly, although there were no significant changes, a trend toward rescue of Tyr/Glu α -tubulin following treatment was identified (manuscript under preparation), and the treatment also rescued some of the behavioural phenotypes of the mice. As mentioned in 1.5, another good candidate biomarker detectable in plasma as well as in the brain, is Neurofilament light chain (NfL). It is certainly, mainly used as a biomarker in neurodegenerative disorders, as it indicates potential axonal damages. Interestingly higher levels of NfL have also been found in neurodevelopmental disorders, such as CDD by the Ulysses Neuroscience team as seen in Figure 2: Plasma Acet/Tot-Tub, NfL and IL-10 are overexpressed in CDD patients and BDNF is downregulated. (manuscript under submission), Additionally, high levels of NfL have also been found in ASD and used as biomarkers of neuroinflammation [325]. We did not find any difference of levels of NfL between the control group and the FXS patients here, but further investigation with a larger sample size may be required as neuroinflammation and altered inflammatory response has been reported in FXS patients [326, 327]. To summarise, given the similar alterations found in CDD and in FXS, there seems to be a possible trend towards microtubule dynamic alteration, in these neurological disorders. Both CDKL5 and FMRP are fundamental proteins in neuronal development, and both interacts with microtubule dynamics, therefore targeting microtubule dynamics in these neurodevelopmental disorders could be a way to find new therapies. Particularly, α -tubulin PTMs have proven promising therapeutical targets in several neurological disorders [195]. Interestingly, the changes in α -tubulin PTMs identified in FXPCs are not the same found in FXS patients; while FXPCs showed a decrease in acetylation and no changes in Tyr/Glu α -tubulin ratio, we found no changes in acetylation in the FXS samples and a decrease in Tyr/Glu α -tubulin ratio in adults. These findings

could reflect the different mechanisms underlying the very different symptomatology linked to FXS and FXPCs individuals, caused by the different impact on brain development of the full-mutation and the premutation. However, it is worth mentioning that at least one study found conflicting results, as they identified a decrease in acetylation in *Fmr1* KO mice rescued by an HDAC6 inhibitor [328]. Therefore, only repeating the analysis on a larger sample size could help confirm these results.

Lastly for the second molecular experiment, to evaluate the potential influence of medications on MT dynamics we performed some exploratory analysis, and we compared medicated and unmedicated patients. Our findings indicated no significant effect, as the Tyr/Glu α -tubulin ratio remained decreased in adults, and there was no change in the Acet/TOT ratio.

Finally, we conducted a series of analysis to investigate any potential relationship between the molecular analysis in the FXPCs group and the language measures in the same group to answer our last research question.

- d) Is it possible to find links between linguistic and molecular measures by combining molecular and linguistic measures to advance development of treatments for FXPCs?

In 1.3, we emphasized the significant role of complex biomarkers—composite markers integrating various biological systems relevant to clinical outcomes[9]—in clinical research, particularly for rare diseases. We proposed that future research should prioritize identifying and combining measures to develop stronger candidate biomarkers to support the advancement of new therapies. Furthermore, we have shown the implications of MT dynamics in neuronal plasticity and NfL in showing potential axonal damage, and that correct functioning of these mechanisms could be fundamental to maintain connections in the major language pathways. Additionally, several molecular mechanisms have already been associated to language performances [329], and here we tried to find potential connections between the plasma-based protein investigated in the molecular experiments and the language measures. We expected to find correlations between molecular and linguistic analysis, however we did not find any noteworthy association in the linear regression models, as only genotype in the model correlating the molecular measures and the response times in the language reception task for objects showed a significant inverse effect, confirming that the FXPCs had higher RTs in the reception task for objects.. This

finding is not particularly revelatory, as it confirms results shown in the previous chapter. Additionally, none of the models reached statistical significance, potentially indicating that none of those factors seems to be a predictor of the language measures considered.

However, the correlations performed to compare molecular and linguistic measures revealed two significant correlations confirming our expectations of a potential relationship between the two measures. Tyr-Glu/tub ratio and errors and dysfluencies were correlated in the object condition of the FXPCs group, and between Tyr-Glu/tub ratio and TOTAL SLD suggesting that a higher inaccuracy and a higher percentage of disfluencies were associated with an increase in Tyr-Glu/tub ratio. Two more correlations were found with Acet-tot/tub in the FXPCs group with the percentage of adjective, and in the CP group with the accuracy in the object condition of the picture naming, and a negative correlation with the retracing number. However, none of them reached statistical significance, but they hold promise for further investigation as they were quite strong correlations.

These findings might suggest specific correlations between biochemical markers and language characteristics, particularly in fluency in descriptive language. The findings in the Acet-tot/tub ratio in the FXPCs group might indicate an implication of lower levels of acetylation associated with more descriptive language, potentially as a compensatory mechanism. While in the CP group decrease in acetylation corresponded to a more disfluent speech, with an increase in retracing (like corrections or hesitations). Acetylation has been found decreased in several neurodegenerative disorders, as mentioned above [313-316], and in all of them this decrease in acetylated α -tubulin was associated with impaired axonal transport, because of the regulatory role believed to be carried out by acetylation in microtubules. These opposite findings might be related to different neural mechanisms engaged by the two groups in language production, particularly that a higher descriptive language might be used by the FXPCs group with lower levels of acetylation engaged in a visual demanding task, to compensate potentially for other cognitive mechanisms, like executive functions challenged by the difficulty of the task [330]. While in the CP group the finding that as acetylation decrease the speech becomes more disfluent, might indicate that the baseline levels of Acet-tot/tub ratio are higher (as our molecular findings in experiment 2 suggests), and therefore as the acetylation decrease in the CP

group, this could indicate a deviation from the norm, leading to a more disfluent speech. However, these correlations were not significant, so the interpretation I only speculative. Therefore, acetylation might be implicated in the neural processes that support speech fluency. However, it is worth reminding that we found a significant difference between levels of Acet-tot/tub ratio in the FXPCs group compared to the CP group, therefore lower levels in the CP group do not correspond to the lower levels of the FXPCs group. The other finding of an increase in Tyr-Glu/tub ratio when the number of Total SLD increase in the FXPCs group, might indicate an implication of certain tyrosination processes in speech fluency. Taken together, these findings suggest a potential link between alpha-tubulin post-translational modifications (PTMs) and mechanisms involved in speech disfluencies during descriptive language. However, no other language measures showed significant correlations with the molecular measures, indicating that the observed correlation may be isolated or have limited impact on other language measures. A key limitation of these correlations is the small sample size, which only included the in-person participants of this study. As such, these findings may not be representative of the broader population and should be validated with a larger sample size. Nonetheless, our analysis shows different implications for the two groups: in the FXPCs group significant correlations were found in both the Acet-tot/tub ratio and in the Tyr-Glu/tub ratio, while in the CP group only the Acet-tot/tub ratio revealed a correlation. This indicates that different mechanisms may be at play in each group, particularly regarding language disfluency.

Chapter 4: Conclusions and Future directions

4.1 Introduction

In Chapter 1 we quoted Shakespeare and his definition of a label, and we moved on to describe the importance of “words” in everyday life and particularly in science. The centre of gravity of this study is indeed “words”. Words because of the importance of definitions in biomarker discovery. Words because in the rare disease field a word is often everything: giving a name to a disease not only means closure to a long and suffered path to a diagnosis, impacting the whole family, but also means validation and most of all means finding a community. Words because “word finding difficulties” can sound the alarm for research and possibilities and can become a biomarker itself. Possibilities that in some cases may or may not occur. As in every language specific letters code for a word, the DNA in the genes code specific instructions for proteins, which in the world of Fragile X Premutation carriers means uncertainty. Anne Skomorowsky in her book “The Carriers” says:

“Carriers are patients-in-waiting [...]. Genes sound the alarm for events that may take place in the future, but in the present, those possibilities may seem completely irrelevant [331].”

With this work we aimed at taking a little further step in helping this community navigate this world of uncertainty through research, by trying to find potential signs that could open the path to further therapeutic discoveries.

Here are the research questions we addressed in this project:

- a) Is it possible to identify potential differences between FXPCs and a control group through language assessment that could indicate prodromal signs of cognitive alterations in FXPCs?
- b) What is the nature of language disfluencies patterns in FXPCs and are these related to a primary language deficit?
- c) Is it possible to identify potential alterations in plasma-based proteins in FXPCs and in FXS? Are these alterations similar?
- d) Is it possible to find links between linguistic and molecular measures by combining molecular and linguistic measures to advance development of treatments for FXPCs? (discussed in Chapter 3).

In the following paragraphs we will outline the answers to the research questions, summarising the main findings, describe our interpretation on the potential mechanisms underlying the findings and finally, explore potential future directions, as well as limitations and strengths of this study.

4.2 Summary of the main findings

We first addressed the first research question

- a) Is it possible to identify potential differences between FXPCs and a control group through language assessment that could indicate prodromal signs of cognitive alterations in FXPCs?

To answer this question, we analysed performance across groups in the three linguistic tasks, and we expected to find group differences across tasks, with the FXPCs group showing poorer performances than the CP group.

In the picture naming task, our findings were mixed and not always significant, so it is difficult to draw definitive conclusions.

In latency, we found differences between groups, to confirm our hypothesis as we observed that FXPCs participants had slower reaction times for actions and objects compared to the CP group, but this was only significant in the automatic scoring modality, which we deemed less reliable in this study design with a dual modality of task administration, due to its susceptibility to environmental noise and confounding variables accentuated in the online modality. The manual scoring, considered more accurate, suggested a similar trend in the objects conditions, though results did not reach statistical significance after correction for multiple comparison. Furthermore, our findings also suggest an effect of task administration modality, where the online modality yielded higher RTs compared to the in-person one, and a higher percentage of planning errors (consisting in the presence of extraneous material and vocalisation before the onset of the target word). These aspects further highlight the potential for confounding variables when tasks are administered remotely. Therefore, while there were indications of slower responses in FXPCs, modality of task administration and scoring modality had an impact on the results, hence more research is needed to clarify these findings.

In accuracy, we found differences suggesting that the FXPCs group was less accurate than the CP group, but the main group effect identified did not yield any significant difference in post hoc comparison. We did find again that modality

influenced performance as the FXPCs group was less accurate in-person, potentially indicating that the FXPCs group felt a higher level of anxiety in that task administration modality. In terms of error patterns, we did not find major differences between groups, however there was a difference in the pattern of the disfluencies type as the FXPCs group showed a higher percentage of circumlocutions type of disfluencies, while the CP group displayed higher percentages of planning subtypes of disfluencies.

In the visual word paradigm task, we anticipated that the FXPCs group would be slower in stimuli recognition. While there was a group effect potentially confirming the hypothesis, post hoc comparisons did not reach statistical significance. However, the results were trending to significance after FDR correction, potentially indicating that the FXPCs group may be at a disadvantage for both objects and actions, but this is only speculative as results are not significant. Additionally, task modality played a role once again, with the FXPCs group showing slower reaction times in the online condition compared to the in-person. In general, FXPCs participants are seemingly slower in the online modality but less accurate in the in-person one, suggesting a difference in task modality administration that needs to be considered in future studies.

In the picture description task our hypothesis of a group difference was confirmed by consistent findings that the FXPCs group had higher percentage of several disfluency types compared to the CP group, confirming previous findings in the literature [7, 8, 332], and that the CP group conversely displayed a richer and more descriptive language. This difference and the higher presence of disfluencies suggests deficits in speech planning that could be linked to mechanisms involved in complex tasks such as working memory or executive function deficits, or to a specific language mechanism. This last aspect will be further addressed with the second research question.

We then answered the second research question:

- a) What is the nature of language disfluencies patterns in FXPCs and are these related to a primary language deficit?

We hypothesised that language disfluencies found in the picture description task would correlate with the time needed to retrieve words in the picture naming and visual word paradigm task. Our hypothesis was confirmed as slower RTs in language production were correlated with a higher percentage of disfluencies in

the picture description task. This potentially suggests that the disfluencies might stem from lexical retrieval impairment where delays in word retrieval might lead to disfluent speech. This potentially contributes to the debate on whether language disfluencies are connected to more general mechanisms such as executive functions, or rather to language specific mechanisms. Here we show a direct connection with tasks tapping directly into language specific mechanisms. However, in this study we did not directly investigate other more general cognitive measures such as working memory or executive function, to confirm this hypothesis.

Finally,, an additional exploratory finding in analysis of language assessments suggests the presence of a word class effect across groups in the picture naming and visual word paradigm task, showing that actions elicited slower RTs compared to actions within group. This finding is present both in the language production and reception task and it is consistent with previous findings in psycholinguistic studies [231, 240] showing that verbs are potentially inherently more difficult than nouns.

Then we moved on to the molecular experiments of the study to answer our third research question:

- b) Is it possible to identify potential alterations in plasma-based proteins in FXPCs and in FXS? Are these alterations similar?

We addressed this question using two distinct molecular experiments. In the first experiment, we anticipated detecting alterations in plasma protein expression in FXPCs compared to CP. Our results supported this expectation; we observed reduced acetylated α -tubulin levels in female FXPCs participants without FXTAS compared to controls. This decrease is consistent with prior findings in literature indicating a decrease in acetylation in several neurodegenerative diseases, including Alzheimer's Disease [291], Huntington's Disease [315], Charcot-Marie-Tooth disease [316], ALS [318], and Parkinson's Disease [292]. Inhibition of HDAC6, a deacetylase involved in microtubule dynamics, has been shown to mitigate symptoms and cognitive deficits in these conditions, highlighting the therapeutic potential of targeting this protein.

No differences in NfL levels were found between groups, suggesting potentially no alterations of this protein in the population of interest.

In molecular experiment 2, we anticipated similar alterations in FXS, however, on the contrary no alterations in Acet/Tub-Total/Tub was found, however a decrease in Tyr/Glu α -tubulin ratios in adult FXS patients was identified. This finding suggests age- related microtubule dysregulation, as no alterations compared to the control group was found in children. These results align with other findings in other neurodevelopmental disorders like CDD found by the Ulysses Neuroscience team, and interestingly GSK-3 β inhibitors have shown potential in *Fmr1* KO mice partially restoring α -tubulin stability, highlighting potential intervention routes.

Lastly, we addressed our fourth research question:

- c) “Is it possible to find links between linguistic and molecular measures by combining molecular and linguistic measures to advance development of treatments for FXPCs”?

To answer this question, we correlated the molecular markers analysis with the language measures. We expected to find connections between the two different types of measures. Correlation with accuracy confirmed this hypothesis, as it showed a significant association with Tyr-Glu/Tub ratio and accuracy in the object condition in the FXPCs group, and between Tyr-Glu/tub ratio and SLD disfluencies, potentially implying a role in tyrosination in speech fluency. However, no meaningful correlations were found between molecular assessments and the latency measures. Taken together these findings suggest a potential role of acetylation and tyrosination in language processing, particularly in the mechanisms leading to disfluencies. This aspect needs to be further investigated in future studies.

4.2.1 Summary of exploratory findings: are there any extra factors correlating with the results?

We performed some exploratory analysis to verify the correlation with factors that potentially might influence the results. Interestingly, in the picture naming task, age was correlated with slower RTs in the CP group in the action condition confirming findings in the literature on healthy aging, but latency performance was not age-related in the FXPCs group, which conflicts with previous findings in the literature [7] [108]. Age was also correlated with performance in accuracy in the CP group in the action condition, indicating greater difficulties in action

retrieval in a healthy aging population, and negatively with FXPCs performance in objects potentially indicating potential compensatory strategies.

CGG repeat size was correlated with performance in latency and larger repeat size corresponded to slower RTs in the object condition. Correlations with the CGG repeat size were found in the literature, were low normal (<25 repeats) and mid-premutation (90-110 repeats) CGG repeat size corresponded to higher rates of language disfluencies [8]. Here we show a link between high-premutation repeat size (100-200) and slower RTs in word retrieval.

In terms of psycholinguistic factors correlating with performance, name agreement was the main consistent variable correlated with performance in both groups for accuracy and latency, while object complexity and frequency showed weaker correlations.

In the language reception tasks, a correlation with age was found in both the FXPCs group and in the CP group in objects, but only in the CP group in actions. No significant influence of CGG repeat size was identified here, potentially showing different mechanisms for lexical access in reception and production.

In the picture description task the CP group again showed moderate correlations between the language measures and age, while surprisingly a moderate negative correlation with age was present in the FXPCs group. This finding is in contrast with previous studies suggesting increases in disfluencies with age [7]. In general, in this task a few moderate correlations with age potentially indicates the presence of compensatory strategies in place to help maintain accuracy level as language difficulties increase with age. While for the CGG repeat size correlations were found with the number of pauses, filled pauses and typical dysfluencies, and negative correlations with the number of syllables and words per minute potentially suggesting the greater the CGG size the higher the number of pauses, filled pauses and dysfluencies, but the smaller the number of words and syllables per minute, suggesting potential difficulties in articulation, but the correlations are too weak to indicate a real effect on language performance.

Interestingly males in the FXPCs group showed slower RTs compared to females, consistent with the presence of more severe symptoms usually detected in males. A gender difference is also present in accuracy: in the object condition females seem to be less accurate, while the opposite pattern is present in the action condition potentially suggesting a different pattern for males and females. However, the number of males who entered this study is too small to draw any

reliable conclusions (in total 9 males and 61 females in the FXPCs group, 9 males and 47 females online, 0 males and 14 females in-person).

In terms of influence of the number of languages spoken, multilingualism yielded mixed effects: while it seems to reduce latency, potentially suggesting improved cognitive processing, it was also correlated with increased errors in FXPCs, potentially alluding to lexical competition between languages in accuracy, which is consistent with previous findings in the literature on multilingualism [255].

In the CP group, no correlations between accuracy and demographic factors were found.

We additionally performed some exploratory analysis in the molecular experiment 1 to investigate potential influence of the demographic factors on the results. No correlation between the demographic factors and the molecular dysregulations, except for a negative correlation between the neurological conditions and the levels of Acet/Tub-Total/Tub in the CP group. It seems like certain neurological conditions contribute to decrease Acet/Tub-Total/Tub levels, as participants with 0 comorbidities had higher levels of Acet/Tub-Total/Tub, while certain neurological conditions like epilepsy and anxiety resulted in lower levels of Acet/Tub-Total/Tub.

4.3 Interpretation of the findings.

In this study our main aim with our first research question was to investigate the presence of differences between groups in three linguistic tasks, and although we found a clear more disfluent pattern in FXPCs compared to CP in the picture description task, other findings in the other tasks are not as strong and somewhat inconsistent. Based on previous literature on PD and other Parkinsonian disorders showing a disadvantage for action naming in these populations [118, 135], we were anticipating a similar outcome for FXPCs, who are at risk of developing a Parkinson-like neurodegenerative disorder, FXTAS. Surprisingly we found this pattern in the picture naming task only in the automatic voice onset detection, which however we believe could have not be the most appropriate for this type of design due to the potential confounding variables online. In both detection modality FXPCs group appears slower than CP group in objects retrieval, but however this result is not significant. This is particularly interesting as potentially might indicate different mechanisms in place for FXPCs, as it hints

at a potential impairment in the temporal lobe [333] instead of the basal ganglia network as identified in PD. Similar findings are present in the language reception task, with a group effect that was not present in post hoc comparison, showing a potential disadvantage for both objects and actions in the FXPCs group compared to the CP group. However again results are not significant, so this is only speculative and further investigations are needed. It is unclear whether our results confirm previous findings in literature on PD indicating a disadvantage for actions compared to objects in FXPCs as well, or if our results potentially hinted at a trend that would distinguish FXPCs from PD, showing a disadvantage for objects instead. Psycholinguistic models have investigated differences between objects (usually corresponding to nouns) and actions (usually corresponding to verbs) extensively [334]. Verbs are considered inherently more complex as they feature one or more agent, requiring greater cognitive demand, hence involving more cognitive resources [135]. Additionally, they are also morphologically and syntactically more complex, as they represent the central element assigning different roles to the other component of the sentence. In contrast, nouns are usually taxonomically organised and connected hierarchically through subordinate and superordinate relationships, which guide retrieval [106]. The lack of structure and organisation in verbs in semantic memory might require more cognitive resources, potentially explaining the within-group differences observed in this study, where performances on the actions conditions were consistently worse compared to objects condition in both language production and reception. As mentioned in 1.4.3, PD patients usually display inefficiency in action word retrieval. This pattern, and in general motor language, seems to be connected to this language network that includes stimuli going from the cortex creating a loop with the BG and subthalamic area and back to the cortex [127]. This mechanism makes sense to explain language deficits in PD, as the BG is affected, however this theory is currently based on indirect evidence on PD and HD, not on direct measures [128]. This theory is consistent with the embodied cognitive framework which challenges the idea of the existence of a separate module for language. The opposite pattern instead, disadvantage of object retrieval, would be consistent with studies finding a disadvantage in AD because of temporal lobe deficits, where semantic objects are processed [106]. The temporal lobe is traditionally associated with the meaning of words, which represent an important part of the

object features [122]. In older individuals word perception is usually associated with bilateral temporal cortex, and in general older individuals tend to recruit a wider brain network compared to younger controls, to compensate for deficits in the main language pathways [126]. Interestingly, difficulties in temporal processing have already been found in adult FXPCs, which would corroborate a potential object disadvantage that even if not confirmed in this study will be fascinating to further investigate in the future. Kim (2014) found alterations in neural correlates of temporal processing in the *when* pathway which is associated with temporal processing and involves the right TPJ, angular gyrus, supramarginal gyrus and the posterior superior temporal gyrus. In this study elevated levels of *FMRI* mRNA predicted the neural alterations which can lead to elevated toxicity, a pattern usually found in FXTAS which can indicate prodromal brain changes [335]. In that study they did not find neural alterations to be correlated with increased CGG repeat size, in contrast we did find a correlation between the CGG repeat size in some of the language measures, particularly in latency. This difference might be related to the different repeat size number of the sample considered, additionally we did not measure levels of mRNA in our participants, therefore we cannot verify whether this finding is present in our study too. Increased numbers of CGG repeats are usually correlated with increased inefficiency of *FMRI* translation and loss of production of FMRP [336]. Elevated CGG repeat numbers corresponds to higher levels of mRNA, which can lead to several dysfunctions involved in the pathogenesis of FXTAS, and the others clinical involvements falling in the umbrella term FXAND, FXPAC. The excess of *FMRI* mRNA is associated with RNA toxicity that leads to the formation of inclusions in cells and tissues, including neurons^{xxxi}, and to mitochondrial dysfunctions which is found in several neurodegenerative disorders such as AD and PD. Both low levels of FMRP and RNA toxicity contributes to the clinical involvements [320] [321]. We did not directly investigate RNA toxicity and FMRP levels in this study, however the correlation with the CGG repeat size might suggest increased inefficiency which can lead to dysfunctions in certain cognitive functions and pathways, including language abilities. As mentioned at the beginning of this paragraph we found a more disfluent speech pattern in FXPCs compared to CP

^{xxxi} To be noted that Intracellular inclusions in central nervous systems is one of the 4 major diagnostic criteria for FXTAS.

that confirmed our hypothesis of differences between groups. Our results of a more disfluent profile in FXPCs are consistent with previous findings in the literature [7]. This finding is particularly important because when symptoms are still not present, language could represent a glimpse of the neural mechanisms associated with FXTAS. The cerebellum has extensively been shown to play a pivotal role in linguistic functions including non-motor language processes. Impairment in verbal fluency tasks have been shown in patients affected by cerebellar lesions, particularly affecting phonetic fluency tasks potentially indicating impairment in phonological retrieval, which can trigger delays and mistakes [337]. The cerebellum seems to be involved in a procedural system related to the correct representation of words and in a variety of non-motor language functions, and accelerated volume decrease in the cerebellum has been found in FXPCs [337]. Particularly, the middle cerebellar peduncle seems to receive projections from several language production regions [109]. Because cerebellar activity is affected in FXPCs, and signs of white matter disease in the middle cerebellar peduncle are one of the diagnostic criteria for FXTAS [183], this aspect could contribute to the language fluency disadvantage found in this study, potentially related to early alterations to the network connecting the cerebellum to the language network. Additionally, the disfluency pattern in picture naming task showed that the FXPCs group used more circumlocutions. The use of circumlocutions is considered a communication strategy in aphasic patients; particularly, semantic circumlocutions are considered strategies used to resolve word retrieval issues, which can be a symptom of anomia, and in non-aphasic patients is simply considered a tip-of the tongue phenomenon^{xxxii} [338]. A higher use of circumlocutions compared to controls was also found in PD patients, particularly in verbs. The authors of that study suggested that patients were able to access semantic memory, but they were using more general language to compensate for difficulties in accessing the target word; as an alternative they proposed that the more specific words are deteriorated first, and the more generic words tends to be easier as they have less semantic properties. The selection of the correct word involves several process such as selection of word meaning, word retrieval with the syntactic and phonological properties,

^{xxxii} The tip-of the tongue phenomenon occurs when one cannot recall a target word but can recall similar words. This phenomenon is often recreated in experimental settings to investigate lexical access.

and finally articulation, as we have seen in the different models of lexical access [132]. It is possible that some stages of this process might be affected in our population and therefore a compensatory strategy is needed, as delays in retrieval in the FXPCs population suggest.

Furthermore, in our second research question we aimed at exploring the origins of the disfluent speech pattern in FXPC. We anticipated that the disfluency in the picture description tasks might be correlated to impairment in the lexical retrieval process investigated with the picture naming and visual word paradigm task. Our findings confirm this hypothesis as we showed correlation between the disfluencies and the lexical retrieval task in word production. The picture naming task specifically investigates mechanisms involved with lexical access, therefore delays in retrieval could be attributed to impairment to this process. Here we show a connection with lexical retrieval delays, and interestingly, several studies have failed in linking impairments to this process to executive functions deficits [106], potentially hinting at a separate process. This might indicate a specific impairment in lexical access for the FXPCs population, which can cause disfluencies and word retrieval difficulties. Additionally, the speech disfluencies pattern identified in the picture description task for the FXPCs group is predominantly composed of repetitions, pauses and filled pauses, which are considered types of disfluencies, indicating difficulties in accessing the target word due to lexical retrieval impairment rather than compensatory strategies [339]. At what stage the lexical retrieval process could be impaired is impossible to tell without direct investigation in this study. ERP studies on the anatomical activation of structures during visual recognition, show that the analysis of the visual cue (visual processing) usually occurs between 50 and 100 milliseconds (ms) after the stimulus is presented, and what is called the pre-lexical stage, the perception of the word starts immediately after. In the brain, frontal areas become available around 180ms while semantic analysis begins at around 200 ms and unfolds at around 500, 600 ms activating frontal and left superior temporal cortex; this phase is considered the lexical stage (when the lemma selection occurs). The post lexical stage (phonological encoding and articulatory process) begins after the first 500ms and develops around 1000 ms [340]. This process involves word picture retrieval, storing phonological information, visual processing and semantic analysis, which includes selection of semantically related competing names. Whether the differences identified with the CP group

found in this study are referring to the pre-lexical or post-lexical level it is impossible to confirm without a direct investigation. However, the correlation with disfluencies might indicate a difficulty at the word selection level stage [341], which is consistent with potential difficulties in semantic memory as the difficulties in object retrieval might suggest.

Concerning the third research question we expected to find plasma-protein alterations in FXPCs and FXS compared to CP. Our hypothesis was confirmed as in the first experiment we found in FXPCs a decrease in acetylation which is consistent with previous findings in neurodegenerative disorders [314-316, 318]. This finding potentially might indicate the early stages of similar disruptions in FXPCs, which might be targeted for interventions. Additionally, in molecular experiment 2 we confirmed again our expectations as the FXS population showed alterations as well, however with a different pattern, potentially indicating different mechanisms at the base of dysregulation in MT alterations in participants with the full mutation.

Finally, with our last research question we anticipated to find correlations between molecular and linguistic measures. Our findings confirm this hypothesis, and this might potentially indicate involvement of these protein-based mechanisms in the processes leading to lexical retrieval. We believe this might be an important finding as these two types of measures combined could indicate stronger signs of alterations that could lead to discovery of targets for treatment development. Our molecular results are based on plasma-based proteins, and previous findings in pre-clinical studies demonstrated that similar changes, measuring these protein alterations in both plasma and in brain tissues, were reflective of what was happening in the brain [187]. However, without a direct investigation we cannot confirm similar alterations in the brain, nevertheless similar dysregulations are reflective of the general health status of the body.

4.3.1 Are these findings signs of cognitive decline?

Whether these findings suggest risk of developing neurodegeneration or simply alterations indicating dysfunctions in FXPCs we cannot indicate with certainty as longitudinal studies would be necessary to confirm this hypothesis. However, our findings demonstrate that non-invasive approaches like language assessments, as well as minimally invasive procedures such as blood draws,

could help pinpoint valuable measures to detect changes in FXPCs even before the development of neurodegeneration. Additionally, previous findings in the literature, show that disfluencies in language production were connected to FXTAS symptoms [109]. These strategies may also shed light on cognitive mechanisms that influence daily activities—especially language and communication—potentially guiding both medical treatment development and tailored language therapies. The language tasks combined with the molecular measure could potentially indicate a molecular modulation of language that could serve in the future to target new treatments. Additionally, the observed potential trend in object-naming disadvantage, which contrasts with patterns typically seen in PD, should be further studies as may serve as an early differentiating marker between these conditions and should be explored further in diagnostic contexts.

4.4 Future directions

The findings of this study show the potential not only for language ability measures in a population at risk of developing neurodegeneration, but also the potential for combining linguistic and molecular measures. This study represents a preliminary investigation exploring the potential for this research path, but further research needs to be done. First, similar language measures should be studied on FXS and be paired with the plasma-based proteins to have a clear picture of potential correlations with the molecular measures and the differences with FXPCs. Additionally, it would be interesting to analyse blood samples from the online population who participated in this study to investigate potential alterations connected to the linguistic measures. Furthermore, more longitudinal studies are needed: administering similar lexical retrieval tasks with different stimuli on the same population at different time points will give the opportunity to longitudinally investigate any potential changes, to point to the direction of cognitive alteration that would confirm the existence of prodromal signs and differentiate between those who bears signs of risk of developing neurodegeneration and those who do not. Similar investigations should also consider any potential changes on the percentage of disfluencies detected in this study longitudinally. This has already been done in a long and comprehensive longitudinal study on female with FXPCs [108] investigating signs of word retrieval difficulty over a period of 8 years. Interestingly their average age range

was 46 years at the end of the study, a very similar average to our study, and signs of language deficits over the years were already detectable, while usually in a healthy population these signs are detectable after the age of 60. Therefore, this aspect should be consistently investigated in a younger population to confirm how early such signs are detectable and what kind of impairment is measurable early. Additionally, it would be interesting to pair the linguistic analysis with electrophysiology, ERP or neuroimaging, to have an idea of the neural network involved which can help to better understand the mechanisms underlying the potential deficit identified. Particularly, it would be fundamental to comprehend the extent of the impact on language processes of the cerebellar involvements of both symptomatic and asymptomatic individuals. Furthermore, in future studies a direct measure of executive functions to compare with linguistic measures would be fundamental to investigate whether the findings of this study really point to the correlation with a specific language function or whether other mechanisms are involved. Finally, any potential future study should repeat the measurements on a larger sample. Despite the differences found in in-person and online modality of task administration, we believe it would be important to keep investigating the potential for online measurement, especially in today's world, where the need for online investigations, especially with the growing need for decentralised clinical trials (DCTs) is becoming increasingly important.

Lastly, it would be fundamental to expand these kinds of language assessments to other languages than English. Out of the over 7000 languages spoken worldwide, most studies are usually run in English, and because languages differ for many structural aspects, it would be necessary in the future to expand clinical studies measuring language impairment in other languages [75]. To the best of our knowledge no language measure has been studied so far in a non-English speaking FXPCs population, therefore it would be fundamental to repeat the measures collected in this study or similar in another languages.

4.5 Strengths and Limitations

To the best of our knowledge this is the first study directly investigating word class difference in FXPCs as a measure of prodromal word retrieval difficulties, found in several neurodegenerative disorders, and correlating it both with disfluencies on descriptive language and molecular analysis. One strength of this

study is to try to fill a knowledge gap in trying to investigate the origins of disfluent language patterns in FXPCs and connecting it to a specific language mechanism. Moreover, both males and females entered this study, while usually language studies on FXPCs includes mostly females. Additionally, another important strength is to combine different measures to make the findings stronger and with a higher potential to be further investigated in future studies as a therapeutic target. Furthermore, the lexical retrieval tasks and the whole experimental session were adapted to a population who might struggle in tasks requiring sustained attention for a long time, and unlike many other similar studies, the psycholinguistic variables in those tasks were perfectly matched between objects and actions, to avoid potential differences due only to the impact of psycholinguistics factors. Moreover, this study attempted to reach a large population worldwide and to make research more accessible for all patients wanting to contribute, extending through Europe, the UK, South Africa, United States, Australia, and New Zealand. We believe this represents a step towards a more inclusive way of seeing and doing research. Lastly, it is a first attempt at combining into a single study FXPCs and FXS; despite the difficulties encountered in recruiting both types of participants, particularly children with FXS, we believe this represent an important proof of concept and step towards the inclusion and consideration of the whole family affected by the *FMRI* mutations in research that could potentially strive to improve their quality of life. This study has also several limitations. The first is the small sample size when modalities are separated due to the extreme difficulties in recruiting participants, particularly in the in-person group, limiting the statistical power of the analysis. Therefore, repeating the experiments with a larger sample size would be necessary to confirm the findings. This is particularly valid for the molecular analysis, which only included the in-person participants, which can therefore only be considered a preliminary analysis to be repeated with a larger sample size. Furthermore, the language reception task, the Visual World Paradigm, is usually used in eye tracking studies to measure word perception shifts of attention and therefore considering several different variables, including eye movements and RTs, to infer conclusions about word reception. However, in our study we only considered mouse click RTs to a verbal stimulus as a measure of word reception, while other variables could be influencing the results, including hearing abilities, motor coordination, and familiarity with the technology.

Additionally, while participants were age matched when the whole sample was considered (in-person and online participants), when separating by modality the in-person controls were younger compared to the FXPCs group; therefore, in future studies the control group should be perfectly age and gender matched to the population considered. Lastly, we did not have the possibility to carry out genetic testing in this study, therefore we couldn't measure CGG repeat size of participants. Subsequently, the control population was recruited based on lack of family history of Fragile X mutations. However, while we cannot exclude with certainty the possibility of the presence of premutation alleles in the CP group, the possibility of this occurrence is about 1 in 300 females and 1 in 850 males in the general population [342], hence 0.13 in 39 females in the CP group who entered this study, and 0.015 in 13 males in the CP group who entered the study.

4.6 Concluding remarks

At the beginning of this work, we mentioned the importance of research in rare disorders and that despite FXS being one of the leading monogenetic causes of intellectual disabilities, there is still no cure or available treatment for it and for Fragile X-associated conditions.

Particularly, FXPCs experience a wide range of symptoms that are still largely unresearched, and they live in fear of developing severe conditions as an effect of carrying a premutation. The lack of reliable and reproducible measures to be used as clinical endpoints or even to detect potential signs that could point to cognitive alterations is one of the main issues to be addressed, especially because most of the time standardised tests are not suitable for the needs of a specific population and lead to clinical trials failure. Therefore, the main goal of this research was to emphasise the importance of finding measures that could be good candidate biomarker, not only by meeting the quality and reproducibility criteria, but also feasibility, which can only be achieved by putting patients at the core of research. Through patient engagement we managed to build a research project that gathered an international community involving non-invasive approaches such as language assessments, and minor invasive techniques such as plasma-based measures. In 1.2 we mentioned how clinical trials are usually based on a risk/benefit assessment and most of the time failures of the trials, particularly in rare disorders, are linked to the use of ineffective clinical endpoints or the lack of scales and usable biomarkers. Here, we have shown the

importance of language studies as a non-invasive and cost-effective measure to detect differences between a population who is at risk of pathological outcomes and a control group. Additionally, we have shown that those language measures could potentially be used not only as signs of cognitive alterations but also could be a step forward to help advance treatment discoveries. We have shown that some of the language measures investigated in FXPCs are correlated with the molecular measures we have considered, such as α -tubulin PTMs. Because those alpha tubulin PTMs are known druggable targets, the linguistic tasks used in this study could represent a valid measure to assess potential changes due to the treatments administered. This aspect emphasises the importance of multidisciplinary studies that put together molecular and cognitive measures to potentially lead the direction for further investigations to develop complex biomarkers, which are fundamentally important, especially in rare disorders. Language measures to investigate cognitive abilities in FXPCs have already been explored in the past, resulting in a linguistic profile characterised by different types of dysfluencies, but many studies failed in identifying the origins of the disfluent speech. Here, we have shown that disfluencies might be related to a specific language process, namely lexical access, which involves several different brain areas and pathways which are often disrupted in cognitive decline. This finding, with further investigations, could potentially help identify people at risk of developing certain condition at a prodromal stage among FXPCs. It could also represent a step forward in better understanding the mechanisms underlying the premutation. Additionally, we have shown that even short, automated tasks, targeted to the patients' needs could potentially be used to reduce –at the minimum– the investigator's intervention, and, the tasks are highly reproducible and adapted to different audiences and ages. Furthermore, we successfully tested the use of online assessments for similar tasks that could be an asset for decentralised clinical trials (DCTs) which are becoming more and more popular. These will enable people from all over the world to participate, making research studies more inclusive. Even though we could not investigate language in FXS in this study, we believe that similar language measures, adapted to children's needs, could be used to investigate the origins of expressive language deficits in children with FXS as well, as similar language specific mechanism which are seldom directly investigated might be disrupted. The molecular investigation of this study also shows promising aspects: as mentioned

in 1.3, one of the biggest challenges in finding feasible measures in neurological disorders is to find alternatives to CSF collection, and the field of blood biomarker discovery has tremendously grown recently. Collecting blood samples is a minor invasive and cost/time effective procedure, and here we have shown that certain plasma-based measures could also be a promising way to differentiate between controls and FXPCs, and potentially represent a good candidate biomarker, particularly to show signs of cognitive decline at an early stage, or simply indicative of alterations happening in the body. The same plasma-based proteins are altered in plasma samples of FXS individuals, potentially representing a usable shared measure to be further investigated in FXS-associated disorders, particularly considering that these measures have been shown to be druggable targets. Whether these plasma-based measures are mirroring what happens in the brain has not been directly investigated in this study but based on past research we believe they could represent a general indicator, and most importantly represent a general indicator of the body health status.

It's important to highlight that the findings of this research are preliminary and represents a contribution to better understand the population of interest and are in no way definite and cannot be used as they are to advance treatment discovery; however, we believe they represent a contribution to advance knowledge on the population and potentially direct further studies to find new targets for treatment discovery.

Finally, we believe that this study shows how patient centricity could be pivotal in leading the way for finding appropriate measures that are not only scientifically appropriate, but also patient-friendly, and that the main objective of similar research should always be not only to improve the quality of lives of the whole family involved, but also to empower patients that should be involved in the research process from the development of the idea to dissemination of results. We hope that similar research with a clear PPI statement will increasingly contribute to inform decision making for all the stakeholders involved in clinical trials.

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Appendix 1: Picture naming task objects

Objects Stimuli	elex1 ³³	ovcjpg	esyll	echar	efric	lnfreq	ecdi
pictures/obj006ant.png	0.88	13915	1	3	0	2.565	2
pictures/obj008anvil.png	0.71	8356	2	5	0	0.693	3
pictures/obj010fishtank.png	0.48	45899	2	8	1	0	3
pictures/obj017baby.png	0.940	18598	2	4	0	5.557	1
pictures/obj037bed.png	1.000	13761	1	3	0	5.136	1
pictures/obj043bicycle.png	0.7	24322	3	7	0	1.792	1
pictures/obj050bone.png	1.000	14370	1	4	0	4.248	3
pictures/obj062bridge.png	0.980	27543	1	6	0	4.205	3
pictures/obj070cage.png	0.918	15117	1	4	0	2.833	3
pictures/obj075candle.png	1.000	8385	2	6	0	2.833	3
pictures/obj087celery.png	0.767	22928	3	6	1	1.386	3
pictures/obj088chain.png	1.000	12912	1	5	1	3.892	3
pictures/obj089chair.png	1	11238	1	5	1	4.92	1
pictures/obj090cheese.png	1.000	12988	1	6	1	3.466	1
pictures/obj095church.png	0.960	34595	1	6	1	5.215	1
pictures/obj105pillar.png	0.465	11413	2	6	0	2.833	3
pictures/obj126dinosaur.png	0.98	12393	3	8	0	1.792	3
pictures/obj127doctor.png	0.837	17528	2	6	0	5.220	2
pictures/obj133dragon.png	1.000	19272	2	6	0	2.303	3
pictures/obj137drill.png	0.628	16254	1	5	0	2.197	3
pictures/obj150faucet.png	0.820	17509	2	6	1	1.099	3
pictures/obj154fire.png	0.960	52543	2	4	1	5.094	3
pictures/obj170fox.png	0.857	16437	1	3	1	2.773	3
pictures/obj175fence.png	0.604	13819	1	5	1	3.434	3
pictures/obj176genie.png	0.977	18559	2	5	1	0.693	3
pictures/obj179girl.png	0.920	15540	1	4	0	6.084	1
pictures/obj180glass.png	0.714	14175	1	5	0	4.984	1
pictures/obj184goat.png	0.959	15302	1	4	0	3.367	3
pictures/obj197hanger.png	0.9	7003	2	6	1	1.099	3
pictures/obj213house.png	0.98	18069	1	5	1	6.409	1
pictures/obj219jack.png	0.854	11170	1	4	1	1.946	3
pictures/obj230knot.png	0.617	12224	1	4	0	2.708	3
pictures/obj232ladle.png	0.55	6129	2	5	0	0.693	3
pictures/obj233ladybug.png	0.67	10682	3	7	0	0	3
pictures/obj243lighthouse.png	0.787	6995	1	3	0	5.170	1
pictures/obj259match.png	0.94	31692	2	10	0	1.386	3
pictures/obj260medal.png	1.000	13078	1	5	0	4.060	3
pictures/obj278neck.png	0.89	21541	2	5	0	2.485	3

³³ As shown in Chapter 2: **Elex1**: Percent name agreement represents the proportion of all valid trials with a codable response. **lnfreq**: frequency based on the CELEX lexical database. **Ovcjpg**: estimates of object visual complexity in the IPNP. **Ecdi**: Objective AoA (Age of Acquisition) the IPNP database established the different AOA from published norms of the American version of the MacArthur Communicative Development Inventories. <https://crl.ucsd.edu/experiments/ipnp/dataquery/queryfull.php>

pictures/obj281nest.png	0.673	5700	1	4	0	4.382	3
pictures/obj285nut.png	0.73	12296	1	4	0	2.89	3
pictures/obj286octopus.png	1	33010	3	7	0	1.099	3
pictures/obj303paw.png	0.674	21167	1	3	0	1.946	3
pictures/obj314piano.png	1.000	19570	3	5	0	3.332	3
pictures/obj339pumpkin.png	1	18960	2	7	0	1.099	2
pictures/obj347rain.png	0.87	20795	1	4	0	4.29	1
pictures/obj354gun.png	0.714	9010	1	3	0	4.605	3
pictures/obj356road.png	0.920	26797	1	4	0	5.521	3
pictures/obj363roof.png	0.939	13178	1	4	0	4.043	2
pictures/obj374salt.png	0.750	8601	1	4	1	3.638	2
pictures/obj390shell.png	0.840	18590	1	5	1	3.850	3
pictures/obj409snake.png	1.000	23761	1	5	1	3.178	3
pictures/obj417thread.png	0.653	13706	1	6	1	2.833	3
pictures/obj431sun.png	1.000	18102	1	3	1	5.030	1
pictures/obj442tear.png	0.500	8908	1	4	0	4.094	3
pictures/obj469tree.png	1	26074	1	4	0	5.257	1
pictures/obj483vase.png	0.94	20221	1	4	1	2.079	3
pictures/obj485violin.png	0.82	8571	3	6	1	1.946	3
pictures/obj510window.png	1	26944	2	6	0	5.303	1
pictures/obj514wolf.png	0.56	15672	1	4	0	2.398	2
pictures/obj519zebra.png	1	36034	2	5	1	1.099	2

Appendix 2: *Picture naming task actions*

Actions stimuli	elex1	ovejpg	esyll	echar	efric	lnfreq	ecdi
pictures/act009fly.png	1.000	13178	1	3	1	4.575	3
pictures/act010wakeup.png	0.364	26195	2	6	0	0.000	3
pictures/act012bake.png	0.478	17724	1	4	0	3.219	3
pictures/act014bark.png	1.000	18031	1	4	0	2.398	3
pictures/act021bowl.png	0.980	17815	1	3	0	1.386	3
pictures/act025buckle.png	0.745	21513	2	6	0	1.609	3
pictures/act033catch.png	0.959	15966	1	5	0	5.268	2
pictures/act038clap.png	0.898	27294	1	4	0	2.565	2
pictures/act039climb.png	0.960	37429	1	5	0	4.533	2
pictures/act046cook.png	0.820	28428	1	4	0	4.290	2
pictures/act047cough.png	0.674	33349	1	5	0	2.565	3
pictures/act050crawl.png	1.000	16855	1	5	0	3.258	3
pictures/act054curtsey.png	0.638	14133	2	7	0	0.693	3
pictures/act062drip.png	0.609	15971	1	4	0	2.398	3
pictures/act067drive.png	0.980	35400	1	5	0	5.389	1
pictures/act072eat.png	0.969	21812	1	3	0	5.670	1
pictures/act073dump.png	0.500	31506	1	4	0	2.890	2
pictures/act087fish.png	1.000	12019	1	4	1	5.100	1
pictures/act099golf.png	0.776	53094	1	4	0	1.386	3
pictures/act105arrest.png	0.512	21843	2	6	0	2.833	3
pictures/act107hatch.png	0.848	19137	1	5	1	1.792	3
pictures/act110hitchhike.png	0.918	26145	2	9	1	0.693	3
pictures/act114iron.png	1.000	13323	2	4	0	1.792	3
pictures/act116juggle.png	0.960	14974	2	6	0	1.099	3
pictures/act120kneel.png	0.826	14002	1	5	0	3.178	3
pictures/act121knight.png	0.692	10071	1	6	0	0.693	3
pictures/act125laugh.png	0.281	22368	1	5	0	5.142	3
pictures/act129light.png	0.900	20907	1	5	0	4.007	3
pictures/act135mail.png	0.771	25541	1	4	0	1.609	3
pictures/act138marry.png	0.717	23413	2	5	0	4.836	3
pictures/act139massage.png	0.960	21386	2	7	0	1.609	3
pictures/act154pet.png	0.980	17815	1	3	0	1.386	3
pictures/act156pinch.png	0.915	17920	1	5	0	2.303	3
pictures/act158play.png	0.960	26095	1	4	0	6.004	1
pictures/act167pull.png	0.792	30784	1	4	0	5.231	1
pictures/act171rake.png	0.980	15121	1	4	0	1.946	3
pictures/act173read.png	1.000	30065	1	4	0	5.924	1
pictures/act182run.png	0.980	17276	1	3	0	6.091	1
pictures/act184salute.png	1.000	15575	2	6	1	1.386	3
pictures/act191serve.png	0.917	32192	1	5	1	5.011	3
pictures/act195sharpen.png	0.717	19312	2	7	0	2.303	3
pictures/act197shave.png	1.000	30336	1	5	0	2.708	3
pictures/act201shower.png	0.878	28383	2	6	0	1.946	3
pictures/act205skate.png	0.776	17040	1	5	1	1.386	2

pictures/act212smile.png	0.958	40153	1	5	1	5.088	1
pictures/act213smoke.png	1.000	17842	1	5	1	3.807	3
pictures/act219spill.png	0.735	23590	1	5	1	2.944	2
pictures/act223spray.png	0.609	23144	1	5	1	2.398	3
pictures/act229sting.png	0.953	23887	1	5	1	2.197	3
pictures/act234surf.png	1.000	20492	1	4	1	0.000	3
pictures/act242teach.png	0.455	25360	1	5	0	4.970	3
pictures/act243tear.png	0.673	27082	1	4	0	4.127	2
pictures/act246throw.png	0.940	24589	1	5	0	5.075	1
pictures/act248tie.png	0.980	23682	1	3	0	4.127	3
pictures/act252type.png	1.000	19194	1	4	0	2.890	3
pictures/act257walk.png	1.000	14385	1	4	0	5.737	1
pictures/act261wave.png	0.958	15853	1	4	0	3.829	3
pictures/act265whistle.png	0.813	19276	2	7	0	2.639	3
pictures/act267wink.png	0.896	20114	1	4	0	2.197	3
pictures/act272write.png	0.920	16774	1	5	0	6.142	1

Appendix 3: Visual Word Paradigm objects

Stimuli features	trial 1	trial 2	trial 3	trial 4
lefttopimage	obj009apple.png	obj193hammer.png	obj169fountain.png	obj229knight.png
righttopimage	obj071cake.png	obj319pillow.png	obj067butterfly.png	obj231ladder.png
leftbotimage	obj042bench.png	obj465tractor.png	obj204helmet.png	obj222puzzle.png
rightbotimage	obj134drawer.png	obj273mouse.png	obj094chimney.png	obj266monkey.png
target_sound	apple.wav	mouse.wav	butterfly.wav	monkey.wav
elex1	1	1	0.86	1
ovcjpg	8241	18988	26607	14511
esyll	2	2	1	1
echar	5	6	4	5
efric	0	0	0	0
lnfreq	3.434	2.944	3.258	3.714
ecdi	1	1	1	1
	trial 5	trial 6	trial 7	trial 8
lefttopimage	obj234lamp.png	obj041belt.png	obj345radio.png	obj327plate.png
righttopimage	obj202heel.png	obj117crown.png	obj327plate.png	obj135dress.png
leftbotimage	obj129doll.png	obj246lion.png	obj226king.png	obj128dog.png
rightbotimage	obj513witch.png	obj496watch.png	obj153finger.png	obj119cup.png
target_sound	doll.wav	watch.wav	finger.wav	dog.wav
elex1	0.98	1	0.915	0.98
ovcjpg	5370	12012	45476	24585
esyll	2	1	2	3
echar	6	3	5	8
efric	1	0	0	0
lnfreq	4.82	4.754	2.565	3.219
ecdi	1	1	1	1
	trial 9	trial 10	trial 11	trial 12
lefttopimage	obj357robot.png	obj446telescope.png	obj174gas.png	obj031basket.png
righttopimage	obj311pencil.png	obj348rainbow.png	obj024ball.png	obj099clock.png
leftbotimage	obj369saddle.png	obj145elephant.png	obj217iron.png	obj188guitar.png
rightbotimage	obj454tiger.png	obj093chicken.png	obj283nose.png	obj338priest.png
target_sound	tiger.wav	elephant.wav	nose.wav	clock.wav
elex1	1	0.98	0.96	1
ovcjpg	4703	25639	15536	32267
esyll	1	1	2	2
echar	4	5	6	4
efric	0	0	0	0
lnfreq	4.407	3.689	3.332	3.258
ecdi	1	1	1	1
	trial 13	trial 14	trial 15	trial 16
lefttopimage	obj066butter.png	obj277nail.png	obj142ear.png	obj342queen.png

righttopimage	obj336potato.png	obj331pool.png	obj189gun.png	obj388shark.png
leftbotimage	obj263mirror.png	obj159flag.png	obj086cat.png	obj472truck.png
rightbotimage	obj284nurse.png	obj246lion.png	obj022bag.png	obj071cake.png
target_sound	butter.wav	lion.wav	cat.wav	cake.wav
elex1	0.959	1	1	1
ovcjpg	9894	16237	18397	28324
esyll	1	1	1	1
echar	3	4	5	4
efric	0	0	1	0
lnfreq	4.22	3.555	4.89	1.792
ecdi	1	1	1	1
	trial 17	trial 18	trial 19	trial 20
lefttopimage	obj467train.png	obj200hay.png	obj157fish.png	obj220jacket.png
righttopimage	obj211horse.png	obj106comb.png	obj222puzzle.png	obj288orange.png
leftbotimage	obj236leaf.png	obj171frog.png	obj133dragon.png	obj123desert.png
rightbotimage	obj267moon.png	obj221jar.png	obj075candle.png	obj409snake.png
target_sound	horse.wav	comb.wav	fish.wav	jacket.wav
elex1	1	0.917	1	0.979
ovcjpg	12019	30351	15082	8732
esyll	1	2	2	1
echar	4	6	6	3
efric	1	1	1	1
lnfreq	5.1	3.761	4.543	4.234
ecdi	1	1	1	1
	trial 21	trial 22		
lefttopimage	obj062bridge.png	obj056box.png		
righttopimage	obj124desk.png	obj225key.png		
leftbotimage	obj163flower.png	obj363roof.png		
rightbotimage	obj259match.png	obj199hat.png		
target_sound	flower.wav	hat.wav		
elex1	1	0.979		
ovcjpg	15082	8732		
esyll	2	1		
echar	6	3		
efric	1	1		
lnfreq	4.543	4.234		
ecdi	1	1		

Appendix 4: Visual Word Paradigm actions

Stimuli features	trial 1	trial 2	trial 3	trial 4
lefttopimage	act183sail.png	act081feed.png	act261wave.png	act113hunt.png
righttopimage	act015beg.png	act046cook.png	act239swim.png	act118kick.png
leftbotimage	act168push.png	act026burn.png	act248tie.png	act024brush.png
rightbotimage	act050crawl.png	act039climb.png	act027bury.png	act142melt
target_sound	push	feed	swim	kick
elx1	1	0.867	1	0.96
ovcjpg	22838	22683	16766	17222
esyll	1	1	1	1
echar	4	4	4	4
efric	0	1	1	0
lnfreq	4.836	4.898	3.871	3.761
ecdi	1	1	1	1
	trial 5	trial 6	trial 7	trial 8
lefttopimage	act169rain.png	act043comb.png	act158play.png	act063drag.png
righttopimage	act153peel.png	act126lick.png	act182run.png	act037chew.png
leftbotimage	act016bite.png	act156pinch.png	act227stand.png	act152bite.png
rightbotimage	act049crash.png	act187scoop.png	act244talk.png	act084file.png
target_sound	bite	comb	talk	file
elx1	0.96	0.959	0.792	0.354
ovcjpg	24562	16924	27347	21375
esyll	1	1	1	1
echar	4	4	4	4
efric	0	0	0	0
lnfreq	3.332	2.303	6.236	3.045
ecdi	1	3	2	3
	trial 9	trial 10	trial 11	trial 12
lefttopimage	act109hit.png	act014bark.png	act207sleep.png	act2013smoke.png
righttopimage	act190sell.png	act222splash.png	act252type.png	act018boil.png
leftbotimage	act085fill.png	act111howl.png	act237sweat.png	act258wash.png
rightbotimage	act243tear.png	act143milk.png	act066drip.png	act120kneel.png
target_sound	hit	splash	sleep	wash
elx1	0.78	0.66	1	0.408
ovcjpg	23139	35117	33733	24257
esyll	1	1	1	1
echar	3	6	5	4
efric	1	1	1	0
lnfreq	4.615	2.398	4.868	4.543
ecdi	1	1	1	1
	trial 13	trial 14	trial 15	trial 16
lefttopimage	act006blow.png	act012bake.png	act161point.png	act165pray.png
righttopimage	act009fly.png	act038clap.png	act178ride.png	act137march.png
leftbotimage	act128lift.png	act091fold.png	act129light.png	act228steal.png

rightbotimage	act059dig.png	act055dance.png	act123knock.png	act002drink.png
target_sound	blow	dance	ride	drink
elex1	1	0.94	0.422	0.875
ovcjpg	19790	30516	18780	25613
esyll	1	1	1	1
echar	4	5	4	5
efric	0	0	0	0
lnfreq	4.443	4.205	4.06	4.868
ecdi	1	1	1	1
	trial 17	trial 18	trial 19	trial 20
lefttopimage	act240swing.png	act095cry.png	act2019spill.png	act260water.png
righttopimage	act200shoot.png	act088float.png	act247tickle.png	act237sweat.png
leftbotimage	act007argue.png	act263weigh.png	act019bounce.png	act163pop.png
rightbotimage	act051cross.png	act274yell.png	act11howl.png	act112hug.png
target_sound	swing	cry	tickle	hug
elex1	1	0.98	0.646	0.84
ovcjpg	18530	22897	18027	16095
esyll	1	1	2	1
echar	5	3	6	3
efric	1	0	0	1
lnfreq	4.043	4.796	1.609	2.485
ecdi	1	1	1	1
	trial 21	trial 22	trial 23	
lefttopimage	act224spread.png	act108hide.png	act035chase.png	
righttopimage	act138marry.png	act210slip.png	act069drown.png	
leftbotimage	act051cross.png	act119kiss.png	act202sing.png	
rightbotimage	act117jump.png	act243tear.png	act164pour.png	
target_sound	jump	kiss	sing	
elex1	0.714	1	0.98	
ovcjpg	15496	31961	23644	
esyll	1	1	1	
echar	4	4	4	
efric	0	0	1	
lnfreq	4.22	4.094	4.369	
ecdi	1	1	1	

Appendix 5: Summary statistics of FLUCAL measures.

[CLAN.pdf \(talkbank.org\)](http://CLAN.pdf(talkbank.org))

morUtts	Categorisation levels	Factor	Mean	N	Std dev	Std error	Median
	FXPCs in person		15.4615	1	6.9596	1.9302	15
	FXPCs Online		15.9608	5	5.9765	0.8369	15
	CP In person		13.5625	1	6.25	1.5625	12
	CP Online		16.9677	3	8.0394	1.4439	15
morWords							
	FXPCs in person		124.7692	13	64.1835	17.8013	116
	FXPCs Online		114.2353	51	57.8718	8.1037	101
	CP In person		119.25	16	60.7108	15.1777	107
	CP Online		129.7742	31	67.066	12.0454	113
morsyllables							
	FXPCs in person		161.2308	13	81.4915	22.6017	151
	FXPCs Online		148.725	5	74.545	10.4385	135
	CP In person		151.937	1	74.448	18.6122	136.5
	CP Online		168.871	3	83.523	15.0012	150
wordmin							
	FXPCs in person		155.871	1	35.538	9.8565	151.154
	FXPCs Online		165.766	5	35.062	4.9097	163.404
	CP In person		165.252	1	40.452	10.1131	168.261
	CP Online		172.6455	31	32.4843	5.8344	169.688
syllablesmin							
	FXPCs in person		203.077	1	41.649	11.5514	196.854
	FXPCs Online		211.184	5	53.966	7.5568	210.857
	CP In person		212.548	1	51.641	12.9103	212.5
	CP Online		227.247	3	43.796	7.8662	220.8
Prolongation							
	FXPCs in person		0.151	1	0.2584	0.0717	0
	FXPCs Online		0.219	5	0.7866	0.1101	0
	CP In person		0.6504	1	1.1915	0.2979	0
	CP Online		0	3	0	0	0
Brokenword							
	FXPCs in person		0	1	0	0	0
	FXPCs Online		0.0632	5	0.2929	0.041	0
	CP In person		0	1	0	0	0
	CP Online		0	3	0	0	0
Block							
	FXPCs In person		1.2718	1	2.0889	0.5793	0.595
	FXPCs Online		0.6699	5	1.1077	0.1551	0
	CP In person		0.3516	1	0.7352	0.1838	0
	CP Online		0.043	3	0.2394	0.043	0
PWR							
	FXPCs In person		0.0285	1	0.1026	0.0285	0
	FXPCs Online		0.0812	5	0.4731	0.0663	0
	CP In person		0	1	0	0	0

	CP Online	0.0104	3	0.058	0.0104	0
PWRRU	FXPCs in person	0.0855	1	0.3081	0.0855	0
	FXPCs Online	0.0812	5	0.4731	0.0663	0
	CP In person	0	1	0	0	0
	CP Online	0.0104	3	0.058	0.0104	0
WWR	FXPCs in person	0.0968	1	0.3489	0.0968	0
	FXPCs Online	0.4201	5	1.0694	0.1497	0
	CP In person	0.1116	1	0.4465	0.1116	0
	CP Online	0.3607	3	0.675	0.1212	0
monoWWR	FXPCs in person	0.0968	1	0.3489	0.0968	0
	FXPCs Online	0.4201	5	1.0694	0.1497	0
	CP In person	0.1116	1	0.4465	0.1116	0
	CP Online	0.3607	3	0.675	0.1212	0
WWRRU	FXPCs in person	0.0968	1	0.3489	0.0968	0
	FXPCs Online	0.4201	5	1.0694	0.1497	0
	CP In person	0.1116	1	0.4465	0.1116	0
	CP Online	0.3607	3	0.675	0.1212	0
monoWWRRU	FXPCs in person	0.0968	1	0.3489	0.0968	0
	FXPCs Online	0.4201	5	1.0694	0.1497	0
	CP In person	0.1116	1	0.4465	0.1116	0
	CP Online	0.3607	3	0.675	0.1212	0
Phonologicalfragment	FXPCs in person	0.123	1	0.2437	0.0676	0
	FXPCs Online	0.0285	5	0.1008	0.0141	0
	CP In person	0.1614	1	0.4862	0.1216	0
	CP Online	0.0514	3	0.2202	0.0396	0
Phraserepetitions	FXPCs in person	0	1	0	0	0
	FXPCs Online	0.0582	5	0.2091	0.0293	0
	CP In person	0.0511	1	0.1395	0.0349	0
	CP Online	0.0638	3	0.2473	0.0444	0
Wordrevisions	FXPCs in person	0.2435	1	0.4198	0.1164	0
	FXPCs Online	0.353	5	0.5626	0.0788	0
	CP In person	0.1177	1	0.3752	0.0938	0
	CP Online	0.1173	3	0.2672	0.048	0
Phraserevisions	FXPCs in person	0.2058	1	0.4121	0.1143	0
	FXPCs Online	0.326	5	0.5797	0.0812	0
	CP In person	0.0612	1	0.1748	0.0437	0
	CP Online	0.0722	3	0.2037	0.0366	0
Pauses	FXPCs in person	1.1882	1	1.7611	0.4885	0
	FXPCs Online	0.8181	5	1.2854	0.18	0
	CP In person	0.6684	1	0.9116	0.2279	0
	CP Online	0.2442	3	0.5713	0.1026	0

Filledpauses	FXPCs in person	3.2246	1	2.8632	0.7941	2.74
	FXPCs Online	3.612	5	2.1298	0.2982	3.75
	CP In person	3.8175	1	2.2601	0.565	3.498
	CP Online	3.4709	3	2.5686	0.4613	3.096
TD	FXPCs in person	4.9852	1	3.5681	0.9896	3.767
	FXPCs Online	4.9997	5	2.989	0.4185	5
	CP In person	4.8772	1	2.9379	0.7345	4.362
	CP Online	4.0196	3	2.9047	0.5217	3.824
SLD	FXPCs in person	1.548	1	2.1636	0.6001	0.685
	FXPCs Online	1.5514	5	2.2782	0.319	0.725
	CP In person	1.1136	1	1.2062	0.3016	0.7195
	CP Online	0.4142	3	0.6996	0.1257	0
TotalSLDTD	FXPCs in person	6.5332	1	5.0773	1.4082	4.452
	FXPCs Online	6.453	5	4.0187	0.5627	5.921
	CP In person	5.9906	1	3.4685	0.8671	5.108
	CP Online	4.4337	3	3.0051	0.5397	4.054

Appendix 6: Summary statistics of all EVAL measures

[CLAN.pdf \(talkbank.org\)](#)

	Categorisation Factor levels	Mean	N	Std dev	Std error	Median
Duration sec	CP in person	42.25	1	18.936	4.7342	41.5
	CP online	45.5484	3	27.345	4.9114	38
	FXPCs in person	46.9231	1	23.865	6.6192	43
	FXPCs online	41.8627	5	21.519	3.0133	37
Total Utts						
	CP in person	13.9375	1	6.5163	1.6291	12
	CP online	14.2903	3	5.8435	1.0495	13
	FXPCs in person	14.7692	1	7.0729	1.9617	15
	FXPCs online	14.4118	5	6.0371	0.8454	14
MLU utts						
	CP in person	13.9375	1	6.5163	1.6291	12
	CP online	14.2903	3	5.8435	1.0495	13
	FXPCs in person	14.7692	1	7.0729	1.9617	15
	FXPCs online	14.3922	5	6.0467	0.8467	14
MLU words						
	CP in person	8.929	1	3.113	0.7783	8.931
	CP online	8.3442	3	2.1971	0.3946	8.267
	FXPCs in person	8.1885	1	1.6908	0.469	7.688
	FXPCs online	7.5947	5	1.972	0.2761	7.222
MLU morphemes						
	CP in person	10.6363	1	3.6938	0.9235	10.285
	CP online	10.094	3	2.4923	0.4476	9.786
	FXPCs in person	9.8152	1	1.9765	0.5482	9.067
	FXPCs online	9.2872	5	2.2671	0.3175	8.769
Freq types						
	CP in person	63.375	1	25.345	6.3363	57.5
	CP online	65.1935	3	23.782	4.2715	63
	FXPCs in person	66.6154	1	28.1411	7.8049	68
	FXPCs online	60.8235	5	23.255	3.2565	58
Freq tokens						
	CP in person	127.75	1	68.408	17.102	113.5
	CP online	127.9677	3	65.960	11.8468	117
	FXPCs in person	127.8462	1	66.303	18.3893	125
	FXPCs online	116.7843	5	62.506	8.7527	107
Freq TTR						
	CP in person	0.5384	1	0.0985	0.0246	0.521
	CP online	0.5489	3	0.092	0.0165	0.548
	FXPCs in person	0.5578	1	0.0894	0.0248	0.546
	FXPCs online	0.5682	5	0.1126	0.0158	0.56
WORDS min						
	CP in person	177.1061	1	41.075	10.2689	182.12
	CP online	176.5449	3	33.013	5.9294	169.78
	FXPCs in person	163.096	1	39.086	10.8405	163.63
	FXPCs online	171.2409	5	37.107	5.1961	167.14
Verbs Utt						
	CP in person	1.4529	1	0.6425	0.1606	1.293

	CP online	1.2405	3	0.2516	0.0452	1.2
	FXPCs in person	1.1671	1	0.3979	0.1104	1.167
	FXPCs online	1.1918	5	0.3316	0.0464	1.182
density						
	CP in person	0.3956	1	0.0511	0.0128	0.397
	CP online	0.409	3	0.0446	0.008	0.401
	FXPCs in person	0.4056	1	0.0486	0.0135	0.404
	FXPCs online	0.4084	5	0.0438	0.0061	0.417
% Nouns						
	CP in person	23.9813	1	3.8117	0.9529	23.874
	CP online	23.1072	3	3.2255	0.5793	22.951
	FXPCs in person	26.8409	1	6.3811	1.7698	27.481
	FXPCs online	22.4969	5	5.043	0.7062	21.656
% Plurals						
	CP in person	21.3495	1	8.8821	2.2205	20
	CP online	21.9294	3	10.542	1.8935	22.222
	FXPCs in person	20.4486	1	8.4299	2.338	23.077
	FXPCs online	20.4901	5	9.8837	1.384	20
% verbs						
	CP in person	17.0623	1	3.3516	0.8379	16.371
	CP online	16.4935	3	2.9794	0.5351	16.667
	FXPCs in person	16.482	1	2.3164	0.6424	16.327
	FXPCs online	16.9896	5	3.289	0.4606	16.547
% Aux						
	CP in person	6.1396	1	2.6249	0.6562	5.8875
	CP online	5.9184	3	3.5409	0.636	5.755
	FXPCs in person	4.7752	1	2.7037	0.7499	5.102
	FXPCs online	5.5328	5	2.2027	0.3084	5.31
% Mod						
	CP in person	0.7312	1	0.9194	0.2298	0.193
	CP online	0.8274	3	0.7602	0.1365	0.787
	FXPCs in person	0.5635	1	0.6136	0.1702	0.442
	FXPCs online	0.8662	5	1.0225	0.1432	0.637
% 3S						
	CP in person	61.3027	1	14.538	3.6347	63.095
	CP online	58.7147	3	17.183	3.0863	57.143
	FXPCs in person	53.6199	1	23.954	6.6438	58.333
	FXPCs online	60.0816	5	14.5114	2.032	60
% 13S						
	CP in person	0	1	0	0	0
	CP online	0	3	0	0	0
	FXPCs in person	0	1	0	0	0
	FXPCs online	0	5	0	0	0
% PAST						
	CP in person	0.5366	1	1.5769	0.3942	0
	CP online	1.3097	3	2.9174	0.524	0
	FXPCs in person	2.2537	1	2.3039	0.639	3.125
	FXPCs online	0.7212	5	1.7283	0.242	0
% PASTP						
	CP in person	2.9167	1	3.4727	0.8682	1.389
	CP online	5.1399	3	5.0329	0.9039	4.878

	FXPCs in person	5.4461	1	5.7855	1.6046	4.545
	FXPCs online	3.8352	5	5.2107	0.7296	0
% presp						
	CP in person	43.3344	1	14.034	3.5086	39.338
	CP online	43.8492	3	13.999	2.5144	42.857
	FXPCs in person	42.0638	1	16.277	4.5147	37.5
	FXPCs online	43.3956	5	14.628	2.0485	41.176
% prep						
	CP in person	9.4826	16	2.6131	0.6533	9.609
	CP online	9.4618	31	3.5369	0.6352	9.836
	FXPCs in person	9.1292	13	2.2592	0.6266	8.163
	FXPCs online	8.2749	51	2.6133	0.3659	8.333
% adj						
	CP in person	2.8038	16	1.6102	0.4025	3.4615
	CP online	2.6415	31	1.3264	0.2382	2.778
	FXPCs in person	2.4359	13	2.129	0.5905	2.717
	FXPCs online	1.8216	51	1.1906	0.1667	1.724
% adv						
	CP in person	4.5442	16	1.9649	0.4912	4.4275
	CP online	4.8248	31	1.5891	0.2854	4.651
	FXPCs in person	5.2155	13	1.9627	0.5444	6.061
	FXPCs online	5.4861	51	2.1625	0.3028	5.31
% conj						
	CP in person	1.1121	16	1.1988	0.2997	0.7525
	CP online	1.1942	31	1.1953	0.2147	0.87
	FXPCs in person	0.7833	13	0.8706	0.2414	0.442
	FXPCs online	1.4586	51	1.3365	0.1871	1.274
% det						
	CP in person	16.577	16	2.5539	0.6385	16.369
	CP online	14.939	31	3.0045	0.5396	14.961
	FXPCs in person	15.225	13	2.8412	0.788	15.217
	FXPCs online	14.320	51	3.6033	0.5046	14.118
% pro						
	CP in person	7.3551	16	3.4067	0.8517	7.081
	CP online	9.8431	31	2.9356	0.5272	9.302
	FXPCs in person	7.9292	13	4.7974	1.3306	6.957
	FXPCs online	9.9657	51	3.5173	0.4925	10.28
noun verb						
	CP in person	1.4694	16	0.3971	0.0993	1.4585
	CP online	1.4706	31	0.4689	0.0842	1.35
	FXPCs in person	1.6642	13	0.4998	0.1386	1.5
	FXPCs online	1.3836	51	0.4344	0.0608	1.294
open closed ratio						
	CP in person	0.9704	16	0.1538	0.0384	0.9485
	CP online	0.9256	31	0.1261	0.0227	0.892
	FXPCs in person	1.0856	13	0.2581	0.0716	1.082
	FXPCs online	0.984	51	0.589	0.0825	0.891
n open class						
	CP in person	60.562	16	30.906	7.7266	54
	CP online	59.612	31	29.698	5.3339	56
	FXPCs in person	63.846	13	33.308	9.2382	58

	FXPCs online	53.627	51	27.745	3.8851	49
n closed class						
	CP in person	66	16	37.813	9.4534	59
	CP online	66.258	31	35.462	6.3693	58
	FXPCs in person	63	13	33.773	9.3672	59
	FXPCs online	61	51	34.944	4.8932	54
retracing						
	CP in person	0.25	16	0.4472	0.1118	0
	CP online	0.3226	31	0.5408	0.0971	0
	FXPCs in person	0.7692	13	1.0127	0.2809	0
	FXPCs online	1.0392	51	1.3851	0.1939	1
repetition						
	CP in person	0.25	16	0.5774	0.1443	0
	CP online	0.5806	31	1.0575	0.1899	0
	FXPCs in person	0.0769	13	0.2774	0.0769	0
	FXPCs online	0.6078	51	1.3428	0.188	0