UNIVERSITÀ
DEGLI STUDI
FIRENZE

DOTTORATO DI RICERCA IN NEUROSCIENZE

CICLO XXXVI

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Non-invasive evaluation of brain activity with functional Near-Infrared Spectroscopy (fNIRS): from normative population to Neurodevelopmental Disorders

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Anni 2020/2023

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Abstract

Neurodevelopmental disorders (NDDs) are neurological conditions affecting brain maturation and causing difficulties in social, cognitive and emotional functioning. Most NDDs are still without an effective treatment, with the lack of clinical biomarkers for monitoring brain function significantly hindering the development of the therapeutic pipeline. Many methodological constraints (mostly, related to the invasiveness of procedures and the need of subjects' compliance) prevent to shift neuroimaging protocols typically used in adults to NDDs populations, limiting functional neuroimaging research in this field. However, emerging evidence suggests that functional Near Infrared Spectroscopy (fNIRS) might be exploited to generate unbiased and reliable measures of developing brain in order to assist diagnosis and drug efficacy studies. Thus, this PhD project aimed to address the research gap in the field, evaluating feasibility, reliability and validity of fNIRS as a novel non-invasive biomarker to measure brain function in under investigated clinical populations of NDDs. To this purpose, a novel standardized fNIRS procedure to measure visual-evoked hemodynamic responses (vHDR) in the occipital cortex has been devised in normative adult population and optimized for children enriching it with high entertaining value. Moreover, test/retest reliability of vHDR metrics has been assessed in the adult population at three different timepoints, proving the robustness of this measure.

This innovative experimental paradigm established a quick and easy strategy for measuring vHDR with fNIRS that maximizes the compliance of young subjects, setting the background for testing its feasibility in clinical samples. Thus, the potential application of vHDR measurement was assessed in two cross-sectional studies comparing fNIRS signal in NDDs groups and control cohorts. A first study was conducted in autistic female (fASD) preschoolers and age-matched typically-developed girls. The aim of this activity was to clarify whether fNIRS could provide a biomarker that might support early clinical assessment of fASD, in parallel advancing the knowledge about phenotype's gender-specificity of this cohort. Then, starting from encouraging data on the preclinical mouse model, the sensitivity of technique was evaluated in a rare metabolic disease caused by genetic deficiency of creatine transporter (CTD), where the translational validation of the imaging biomarker might open the way to its use in drug efficacy trials. In both clinical samples the feasibility and validity of vHDR have been robustly assessed.

These results might expand the frontiers of fNIRS technique, pointing to the analysis of vHDR as a valuable tool for assessing brain function in both observational studies and clinical trials including NDDs patients.

1 Introduction

1.1 Basic principles of functional Near-Infrared Spectroscopy (fNIRS)

1.1.1 Technical principles

Near Infrared Spectroscopy (NIRS) is a non-invasive optical imaging technique that uses absorption of near-infrared light to quantify changes in concentration of specific chromophores. Since the biological tissue is relatively transparent at wavelengths belonging to the near-infrared light spectrum (approximately 600-900 nm), NIRS is able to provide a measurement of changes in the concentration of oxygenated (OHb), deoxygenated (DHb) and total (THb) hemoglobin, detecting the degree of attenuation of emitted light. These modifications reflect changing in local haemodynamics and oxygenation of several tissues including the brain (Lloyd-Fox et al., 2010, van de Rijt et al., 2018). Thus, functional Near Infrared Spectroscopy (fNIRS) is capable of providing an indirect measurement of neuronal activity based on the hemodynamic changes in cortical structures, in line with the principle of neurovascular coupling: as a rule, in fact, brain activity leads to an increase in oxygen consumption, accompanied by an increase in cerebral blood flow (Logothetis et al., 2004). Neurovascular coupling typically induces an increase in THb and OHb concentration as well as a decrease in DHb concentration. This pattern of variations in Hb concentrations over time defines the Hemodynamic Response Function (HRF).

Physics principle of the technique is the modified Beer Lambert's law that highlights the correlation between the modifications of light attenuation and concentration of chromophores according to different and specific absorption coefficients (Strangman et al., 2002):

$$\Delta A = a \cdot \Delta c \cdot L \cdot DPF$$

where:

- *A is the absorbance*
- *α is the molar attenuation coefficient or absorptivity of the attenuating species*
- *c is the concentration of the attenuating species*
- *L is the optical path length*
- *DPF is the Differential Pathlength Factor, factor dependent on the type of tissue and wavelength.*

Over time, different models for measuring the hemodynamic response using NIRS have been proposed (Hielscher et al., 2002):

– Continuous Wave (CW);
– Time-Resolved (TR);
– Frequency-Resolved (FR).

To date, the CW technique appears to be more widely used than the others (Blasi et al., 2007). It involves the use of multiple source-detector pairs (channels), capable of providing a two-dimensional mapping of the cerebral hemodynamic response using a signal analysis technique known as "optical topography" (Watanabe et al., 1996). Each detector records the light signal coming from a small group of nearby sources: to identify the exact location of the source originating the signal, the software will then perform a single frequency intensity modulation. The arrays of multiple NIR sources and detectors enable a broad area of the cortical surface to be sampled with a minimal number of optical fibers, and a two-dimensional map of surface brain activity to be generated. To identify the source associated with a given detected signal it is necessary to either illuminate each source sequentially, or modulate each source at a unique frequency, and then distinguish the signals using lock-in amplifiers or a Fourier transform in software. Measurements on all detectors can be recorded at a rate of several Hz, enabling the time-course of the typical hemodynamic response to be adequately sampled. Nevertheless, the main limitation of CW technique is the difficulty to derive absolute values of chromophore concentrations due to a non-linear spatial distribution of NIR light into tissues.

To evaluate changes in chromophore concentration in non-arbitrary units, it is necessary to determine the average pathlength of the diffusely reflected light through the employ of a source of short (picosecond) laser pulses and a fast time-resolved

detector. Thus, the TR technique offers the best spatial resolution, thanks to the measurement of the time of flight (TOF) of single photons (Davies et al., 2015). Unfortunately, TR systems require longer data acquisition times than CW systems to obtain a comparable signal to noise ratio (SNR).

Finally, the FR systems enable the average path length to be determined using an intensity-modulated source and a device that measures the phase delay of the transmitted signal (Minagawa-Kawai et al., 2008). Such systems do not rely on photon counting, and therefore offer a greater SNR and dynamic range than time-domain methods. However, they provide less equivalent temporal information unless multiple frequencies are employed, and operate less efficiently at very low intensities. Many optical topography systems have been built based on both time-domain and frequency-domain technologies. However, their disadvantage compared to CW systems is their comparatively high cost and less efficient sampling rates (particularly for photon-counting systems).

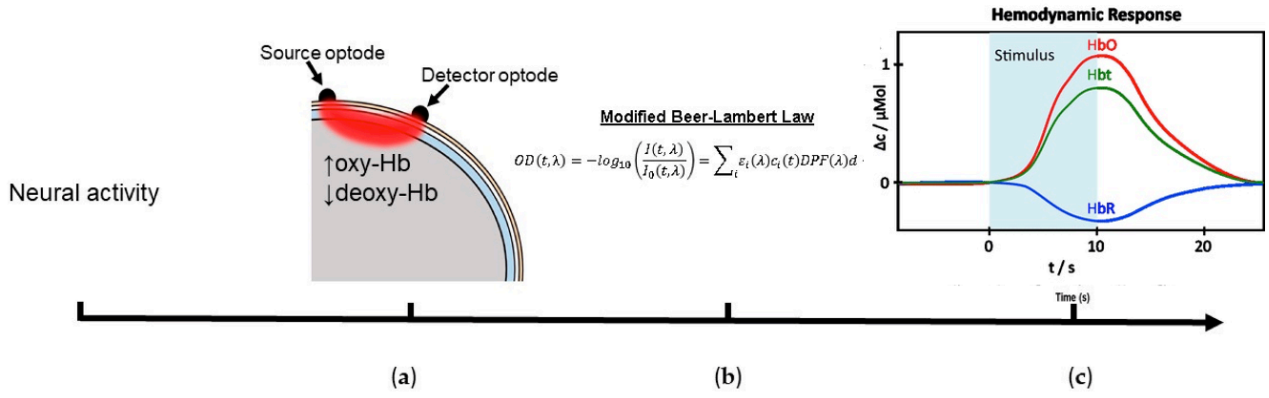

Figure 1.1 Schematic representation of signal recorded by fNIRS (modified from Dans et al., 2021); panel (a) corresponds to schematic representation of scalp placement of a fNIRS channel made by a source and a detector; panel (b) mathematically represents the Modified Beer-Lambert Law; in panel (c) typical pattern of HDR measured by fNIRS with an increase of OHb and THb and a deflection of DHb; nevertheless, non-canonical response are reported to in literature (for a detailed description see next paragraph).

1.1.2 Hemodynamic response: variability and interpretation

In addition to experimental models used (i.e CW, TR and FR techniques), fNIRS studies need to take into account also the variability of HRF over time and brain areas, especially in the developmental age.

The typical pattern of response to a stimulus – at least in the adult population – is characterized by an increase in OHb and THb with a parallel decrease in DHb, as previously discussed. However, numerous studies have demonstrated how the response pattern in children can differ from that of adults, highlighting in particular the lower relevance that DHb modifications have in childhood and thus considering the changes in OHb and THb as the most relevant analysis parameters (Lloyd-Fox et al., 2010). It has been hypothesized that this deviation from the typical pattern may depend on age, the brain area under study and the experimental conditions (sleep/sedation or wakefulness), as well as on the physiological excess of superfluous synaptic connections in infancy (Lloyd-Fox et al., 2010; Marcar et al., 2004). Moreover, fNIRS and fMRI studies show that the shape of the hemodynamic response changes with age in different brain areas (and perceptual or cognitive tasks): canonical (i.e., statistically significant increase in OHb and decrease in DHb as compared to baseline) and inverted (statistically significant decrease in OHb and increase in DHb as compared to baseline) responses have both been reported in the literature. Non-canonical responses, especially the "inverted response", are not straightforward to interpret (i.e., whether they are suggestive of vascular problems). The inverted response is often observed in newborns and infants younger than one year old and it could probably reflect a physiological process of brain maturation, gradually then normalizing to a more canonical shape. For example, infant fNIRS studies investigating auditory perception in the temporal cortex have demonstrated both inverted and canonical responses (Telkemeyer et al., 2009), while later in infancy, from 3 months onwards, infants' temporal cortex increasingly often shows a canonical response (Emberson et al., 2017). Indeed, the HRF relies on a complex interaction between the vascular system, neurons and glial cells, all of which undergo considerable maturation throughout infancy and childhood, possibly with significative topological differences between one brain area to another. Moreover, peak latency (i.e., time to reach the maximum of the HRF) has also been reported to decrease through infancy (Issard et Gervain, 2018). The HRF also varies with the tested sensory modality or cognitive function and their developmental stage, but also with the arousal level of the participants (i.e., asleep vs awake). Focusing on the HDR to social stimuli

(i.e., faces or infant-directed speech), typically investigated in the posterior temporo-parietal region several studies have showed a wide range of response variability to the same stimuli with age. At 4 months of age, infants display a delayed but canonical response to social stimuli, and this response becomes faster during the first two years of life, but inverted responses have also been observed at similar ages in similar regions of interest (ROIs) (Lloyd-Fox et al., 2018). At 5–6 and 7–8 months, a series of different faces presented from different points of view evoked a canonical response, whereas a series of the same face presented from different points of view evoked an inverted response (Kobayashi et al., 2011).

Finally, the HRF seems to be further influenced by the complexity of the stimuli used and the experimental design (i.e., a repeated presentation of identical stimuli vs paradigm with alternate conditions), even within the same age or brain area. Indeed, in developmental psychology it is well known the effect of stimulus complexity/familiarity on infants' behavior, such as looking time, can considerably affect response trough a U-shaped function. Another relevant question is how different degrees of complexity or familiarity may change the type of processing mechanisms triggered. Each of these factors has a different impact on the hemodynamic response, and they interact with one another, justifying the importance of considering many variables when interpreting NIRS results, especially non-canonical ones.

1.1.3 Methodological considerations

While the first studies carried out with NIRS used a few channels (from 1 to 3), awareness has gradually been acquired of the advantages of using multiple channel instrumentation (covering whole brain), since capable of providing increasingly precise spatial localization of the recorded signal (van de Rijt et al., 2016). The increase in the number of channels involves, however, an increase in the size and weight of the headgear, sometimes generating movement artefacts – especially in children – linked to poor tolerance of the bulky instrumentation and high dropout rates compared with adults (Lloyd-Fox et al., 2010). Indeed, in first reports the proportion of infants excluded in a NIRS study due to unsatisfactory data was approximately 40% (Lloyd-Fox et al., 2010). The prerequisites for NIRS headgear for infant studies are that it must be comfortable, lightweight, easy to position in a very short space of time and must provide reliable continuous optical measurements from all channels. At practical level, a strong effort has been done for optimizing headgear design, in order to develop an efficient

and comfortable method by which to attach multiple source and detector optodes to the infant head. To date, several commercial fNIRS instrumentations are available with customized equipment tailored for developmental age (i.e. blunt tip optodes for newborns and infants) (Kassab et al., 2015).

An additional issue to consider for scalp-based technique such as fNIRS and EEG, is the lack of direct structural information: in order to spatially assess the cortical source of the measured signal, the correspondence between each scalp location and its underlying cortical surface must be established. In particular, two factors are critical in determining the sensitivity of each fNIRS channel to the underlying cortex:

- the location of sources and detectors;
- the distance between sources and detectors.

First, the placement of channels according to the 10-20 international reference system allows an effective correspondence to anatomical regions in the adult brain (Okamoto et al., 2004). Based on this scalp-anatomy correlation, several software tools have been developed to facilitate fNIRS researchers in designing the montage, based on the cortical regions of interest, i.e., FOLD software (Zimeo Morais et al., 2018) that defines optimal positions of optodes. Some studies systematically investigated the validity of correlation between external anatomical landmarks, conventionally the 10–20 sensor placement system and internal cerebral structures also in newborns, infants and children (Cai et al., 2021), sharing tailored template and atlases (Fu & Richards., 2021; Kabdebon et al., 2014).

Regarding the optimal distance between optodes, the depth reached by the photons is approximately half the separation between source and detector (Fukui et al., 2003): while short separations allow to sample the superficial layers right under the scalp (Emberson et al., 2016), such as skull and cerebrospinal-fluid, longer separations allow to probe deeper regions such as cortical regions of the brain, at the expense of reducing the SNR. Overall, for correct positioning of the optodes on the cranial surface it is necessary to consider that the separation between source and detector should be, at least, double the distance between the skin surface and the cortical surface. In the adult, therefore, the separation between the channels will be between 40 and 50 mm (Okada et al., 1997); in children or neonates – given the lower thickness and greater translucency of the surfaces – the distance may be limited to 20 – 30 mm (Cai et al., 2019). The optimal separation distance, however, varies based on the intensity of the light source, the age of the subject under study and the location of the cortical area of interest (Beauchamp et al., 2011). It’s important to take into account that working with

subjects at early developmental stage (infants and children) needs practical considerations regarding methodological issues, significantly different from rules used on adults (Lloyd-Fox et al., 2010), that will be discuss deeply in following paragraphs.

1.1.4 fNIRS compared to other functional neuroimaging techniques

Non-invasive assessment of human brain activity is a major challenge, especially in developmental age. This paragraph aims to provide a brief survey of multiple functional techniques to assess brain activity highlighting advantages and disadvantages of fNIRS. Scalp-based neurophysiological techniques such as electroencephalography (EEG) and magnetoencephalography (MEG) offer a direct measurement of neuronal activity but present sensitivity, localization, and resolution issues; therefore, they are rarely used for functional brain imaging research, at least in developmental age. In comparison to EEG, fNIRS uses a similar experimental setting, is less susceptible to data corruption by movement artifacts and yields a more highly spatially resolved signal (Lloyd-Fox et al., 2010), while presenting lower temporal resolution (Luck, 2005).
All the other methods of functional brain imaging rely on proxy measures of neuronal activity that are related to local blood flow, oxygenation, or metabolism (Raichle 1998). The most common of these functional brain imaging techniques is functional magnetic resonance imaging (fMRI), providing like fNIRS measurements of hemodynamic responses linked to neurovascular coupling (Eggebrecht et al. 2012; Hillman, 2014). fMRI Blood Oxygen Level Dependent (BOLD) is a measure of the levels of DHb; decreases in DHb lead to an increase in signal which is termed "positive BOLD" and typically interpreted as an increase in neural activity. fNIRS takes advantage of the different optical absorption spectra of OHb and DHb to measure relative changes in each chromophore across the cortex using different wavelengths of near-infrared light. Thus, fNIRS gives back a more complex signal that reflects hemodynamic variations in the arterial, venous and capillary district (Lloyd-Fox et al., 2010), enabling the assessment of both blood oxygenation and volume changes. Moreover, due to fast sampling rate, fNIRS appears to have a higher temporal resolution compared to fMRI. Further advantages of NIRS compared to the fMRI method concern the silence of the equipment, the lower sensitivity to movement artefacts and – consequently – the possibility of carrying out studies in a more ecological setting (Agbangla et al., 2017). The creation of a comfortable context capable of guaranteeing a good margin of movement for the subject is particularly useful in developmental age, and even more in subjects suffering

from Neurodevelopmental Disorders (NDDs) including Attention Deficit and Hyperactivity Disorder (ADHD), and Autism Spectrum Disorder (ASD), in which the prolonged immobilization required to perform fMRI would be hard to obtain (Zhang & Roeyers, 2019).

Nevertheless, there are also some limitations of fNIRS technique to discuss. The main disadvantages of NIRS compared to fMRI are the lower spatial resolution (approximately 1 cm) and the inability to carry out an accurate structural analysis of the brain components, especially with regard to the deep regions (Aslin, 2012). Moreover, a limit shared by both NIRS and fMRI compared to technique that directly measure neuronal activity like EEG and MEG is the so-called hemodynamic delay: between the neuronal electrical activation and the hemodynamic modifications there is inevitably a time interval – on average 5 or 6 seconds – which must also be taken into consideration during the design of the study, in order to choose the adequate duration of stimuli and intervals within them, in order to allow return to baseline (Cui et al., 2010).

To conclude, compared to other neuroimaging techniques, fNIRS stands out for its portability, non-invasiveness, experimental flexibility to more ecological setting but also reliability of signal and robustness to motion artifacts. Thus, given its methodological advantages, fNIRS is an ideal neuroimaging tool to study neurodevelopment. It holds the potential to provide new insights about neural mechanisms, suggest biomarkers for screening infants and children for developmental disorders, and serve as an objective measure for the impacts of interventions.

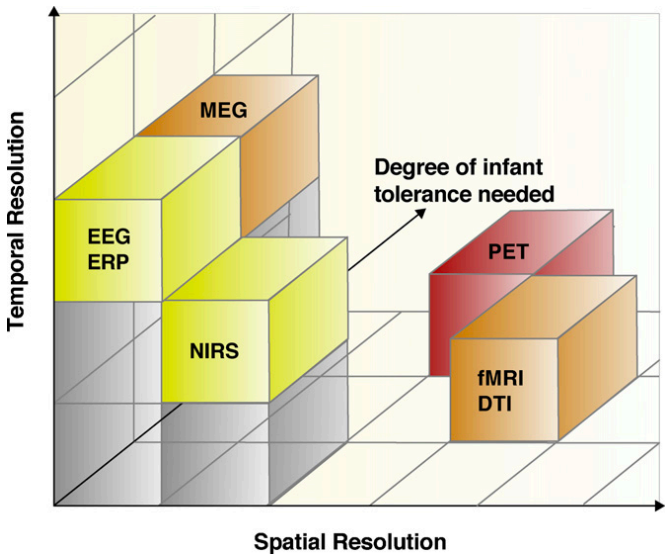

Figure 1.2 Graphical representation of fNIRS technique compared to other neuroimaging methods in terms of spatial (x-axis) and temporal (y-axis) resolution (from Lloyd-Fox et al., 2010).

1.1.5 Experimental paradigm

When selecting an experimental design for fNIRS, researchers must consider a range of factors, including the statistical power of the protocol, the duration of the experiment, and whether the design provides the flexibility to study the effect of interest. FNIRS could be used both for measuring the cortical activation on a specific ROI in response to a stimulus (task-evoked study) or for measuring hemodynamics emanating from spontaneous neural activity, thus calculating spontaneous functional connectivity of the cortical network under exam (resting-state functional connectivity study). These two experimental paradigms are briefly discussed below.

For signal analysis, it is very important to consider some types of noise and artifacts that can constitute relevant confounding factors if adequate containment techniques are not adopted. First of all, some studies have highlighted how the subject is often able to "anticipate" the onset of the stimulus (Csibra et al., 2001) leading to an "anticipatory effect". To minimize this potential measurement error, the length of the control period (baseline) can be varied or the appearance of the stimulus randomized, so that the pattern of presentation of the stimuli is not predictable (Lloyd-Fox et al., 2010). In addition to the anticipatory effect, further confounding factors are given by three different types of noise (Lloyd-Fox et al., 2010):

- Noise generated by the instrumentation;
- Low frequency physiological oscillations;
- Movement artefacts: especially relevant in children, who are often unable to limit their motor skills.

Regarding the noise generated by the instrumentation, it is sufficient to apply low-pass filters, capable of allowing the passage of only signals with a frequency lower than a threshold, while attenuating those with a higher frequency (Koh et al., 2009).

Physiological oscillations are represented by breathing, heart rate, Meyer waves (low frequency arterial pressure oscillations): they can mimic the frequency and amplitude of the hemodynamic response to the stimulus, constituting an important source of error (Orihuela-Espina et al., 2010). To limit these confounding factors – first and foremost cerebrovascular oscillations – numerous studies highlight the importance of an experimental model which envisages the presentation of the stimulus at a frequency such as to minimize as much as possible the coupling between the functional hemodynamic activation and the aforementioned spontaneous oscillations. Furthermore, fNIRS recording also reflects surface vascular signals arising from non-

cortical sources (e.g., in the skull, skin, dura, and other tissues located between the sensors and the brain). To remove this kind of measurement bias, the use of a reference channel, called short-channel, has been proposed: it is characterized by a source – detector distance of about 1 cm, much lower than the commonly applied one, which allows the surface activity to be electively detected and subtracted from the fNIRS signal (Gagnon et al., 2012). Alternatively, a mock stimulus can be included into the experimental design serving as an inter-trial control event in order to verify HDR specificity of stimulus vs baseline.

Movement artifacts can instead be contained thanks to algorithms capable of eliminating data blocks referring exclusively to the signal generated by this type of noise, even with specific pipelines for infants and children (Di Lorenzo et al., 2019).

1.1.5.1 Task-evoked functional experiments

For task-evoked study, the experimental paradigms most commonly employed in fNIRS literature are block and event-related designs,

Block Design (BD) is constituted by blocks of different stimuli, lasting between 4 and 30 seconds, presented to the subject. Each block is followed by a control condition of adequate duration to guarantee the return of the HRF to baseline. Due to the repetitive presentation of the stimulus, only the HRF of the entire block can be detected and not of individual stimuli (van de Rijt et al., 2018). The BD appears to be the most widely used experimental model, apart from a few rare exceptions.

Event-related Design (ERD) is made of short stimuli, lasting between 1 and 4 seconds, presented in succession, with greater rapidity than the presentation time of the individual blocks in the BD. The faster presentation of stimuli reduces the time required for data acquisition, thus allowing to increase the total number of recordable events. However, for the statistical analysis of data obtained with this experimental model, more complex algorithms are required than those needed for BD (van de Rijt et al., 2018).

Whatever the model adopted for the study, the hemodynamic response recorded following the presentation of a stimulus must always be related to a control condition (baseline). Finally, during a task-evoked fNIRS study it is useful to take into account two other fundamental aspects:

- Specificity of the stimulus: numerous studies have demonstrated how the presence of any stimulus in itself leads to an increase in arousal/attention, which could cause the activation of a response recorded by some NIRS channels; this response, however, would not be strictly linked to the stimulus itself, but rather to a systemic gain in the hemodynamic response that follows the increase in

arousal compared to the baseline condition, thus being able to generate a non-negligible measurement error (Aslin, 2012).

- Regional selectivity: not all stimuli activate cortical regions according to the patterns predicted by the experimental design. In fact, there is often a notable dispersion of activation across numerous brain areas, and the signal is much more widespread than expected (van de Rijt et al., 2018).

1.1.5.2 Resting-state functional connectivity analysis

In addition to task-evoked functional experiments, fNIRS is also widely used to assess resting-state functional connectivity (FC) across multiple brain regions. Functional connectivity concerns the investigation of the correlation in slow signal changes (<0.1 Hz) between different parts of the brain. The sampling rate of typical fNIRS systems is 10 Hz, which provides ideal data for connectivity measures with a reduced risk of aliasing of activity with higher frequency (e.g., heart rate at approximately 1 Hz frequency) into the lower frequencies (<0.1 Hz), when compared to fMRI. Several studies showcase the flexibility of fNIRS for the assessment of cortical connectivity. The method has been validated using simultaneous fNIRS–fMRI measurements and is also suitable for infants (Bulgarelli et al., 2018) with no need of sedatives, offering a promising window to study developing brain (Hu et al., 2020).

In terms of analysis methods, classical approaches have been extended from fMRI to fNIRS such as the seed-based correlation method that evaluates the correlation between a seed region and other regions (Lu et al., 2020). The seed can be an fNIRS channel and the correlation or the coherence at different frequency bands can be evaluated between all possible pairs of seed-channels (Sasai et al., 2011). Growing evidence is rising regarding shared toolbox dedicated to optimize fNIRS signal analysis (Hou et al., 2021)

1.2 Application of fNIRS in cognitive neuroscience: from developmental age to adult brain

The fNIRS technique is developing rapidly over these years and the researchers of different areas are engaging in better understanding the functionality of the human brain. The main fields of fNIRS application are related to neurology, psychiatry, psychology, education, cognitive and social neuroscience, and many more. These include physiological brain development and ageing, but also pathological conditions such as Traumatic Brain Injury (TBI), neuro-motor (like stroke or neurodegenerative diseases) anxiety and mood disorder, schizophrenia, and so on in adults as well as NDDs in children (briefly summarized in following paragraphs, both in adult and in developmental age).

Notably, beyond the single participant studies reviewed above, one of the most exciting and rapidly growing areas of fNIRS is hyperscanning, where signals are recorded from two or more participants simultaneously, thus monitoring brain activation during natural interaction behaviors. By simultaneously recording from more than one brain, not only intra- but also inter-brain neural relations can be investigated (Czeszumski et al., 2020). It is common to use functional connectivity (FC) to quantify this synchronization. Depending on the paradigm and hypothesis, one could look at FC between the same region in different subjects, or different regions within different subjects – i.e., synchronization between Broca's area in a subject that is speaking compared to and Wernicke's area in a subject that is listening. It's worth noting that, unlike fMRI and EEG, fNIRS allows to measure cortical activity without restraining the subject's usual behavior in a social interaction, allowing for a higher ecological validity of the measurements. Possible applications to neuroscience of hyperscanning with fNIRS include, but are not limited to: mother-child interaction (e.g. Azhari et al., 2019), social self-perception (e.g. Balconi & Vanutelli, 2016) and atypical pattern in clinical samples (Kruppa et al., 2021).

Moreover, at least in adulthood, neuroimaging techniques are becoming important tools also for rehabilitation research. Functional neuroimaging techniques can be used to determine the effects of brain injury or disease on brain systems related to cognition and behavior, and to determine how rehabilitation changes cortical circuits. In particular, fNIRS has been applied to monitor brain plasticity after neurovascular events, both as a single technique or in more complex Brain Computer Interface (BCI) models.

1.2.1 fNIRS studies in adult subjects

Application of fNIRS technique in adults are wide, including the analysis of pure motor control during simple (i.e., finger tapping) and complex task (i.e., walking), but also more complex cognitive functions, ranging from normative population to the identification of atypical cortical HRF in acute and chronic neurological disorders. Moreover, lots of fNIRS papers focused on psychiatric disorders and their impact on cortical brain activation, identifying possible diagnostic landmark as well as putative markers for clinical monitoring.

1.2.1.1 fNIRS application in neurology

There is a considerable wealth of fNIRS literature showing that the regulation of motor functions in adult subjects is provided by primary cortical regions with the important contribution of higher multi-modal areas. Several multichannel fNIRS studies, indeed, have confirmed a reliable signal with a regionally specific response (Leff et al., 2011): motor stimuli precipitate typical hemodynamic responses over the primary motor cortex (M1) and maximal responses are observed in the hemisphere contralateral (versus ipsilateral) to the performing limb. However, the magnitude of the response may be modulated by the frequency, intensity, and/or complexity of motor stimulation, possibly reflecting the demand for additional neuronal recruitment. In healthy subjects, fNIRS studies have delineated the role of the primary sensorimotor cortex in walking, and of the prefrontal cortex (PFC) in realistic complex motor tasks of everyday living such as maintaining attention and concentration required for running (Harada et al., 2009).
Consistently with other neuroimaging studies (Kato et al., 2002), fNIRS research suggested that stroke patients suffer an impaired contralateral M1 activation, up regulation of ipsilateral motor activation, and longitudinal improvement in laterality toward contralateral M1 activation following rehabilitation (Takeda et al., 2007). Similar evidence came out from studies about Traumatic Brain Injury (TBI), suggesting the promising role of the technique in monitoring changes in cerebral hemodynamics that might mirror cortical functional reorganization between the acute and the chronic phase (Bhambhani et al., 2006).
Regarding chronic neurodegenerative disorders, fNIRS has been applied to Multiple Sclerosis (MS), Parkinson Disease (PD), and, mostly, Alzheimer Disease (AD).

Cerebral oxygenation in patients with AD was frequently investigated by means of verbal fluency tasks (VFT) showing impaired hemispheric lateralization as well as reduced OHb concentration increases (reflecting diminished cortical activation) within different brain regions, especially the dorsolateral PFC and parietal cortex (Arai et al., 2006). Moreover, fNIRS appears to have a prognostic role since many studies propose its use to differentiate dementia-related conditions and to identify Mild Cognitive Impairment (MCI)/AD prodromal stages. Indeed, fNIRS allows us to monitor through the PFC the decline of executive functions and visuospatial abilities, which is an early symptom of these pathologies (Ung et al., 2020).

Most of the studies in PD are focused on walking and dual-task conditions to investigate the extent of PFC motor vs. executive and cognitive dysfunction. Indeed, the underlying hypothesis is that PFC compensates for the motor impairment, hence cortical activation can be considered as an overall index of cognitive load. Within the context of rehabilitation, the promotion of more localized cortical activation associated with executive-attentional functions could be a viable way to activate this compensatory mechanism (Stuart et al., 2019). Moreover, a considerable number of articles about PD uses fNIRS to evaluate the effect of Deep Brain Stimulation, investigating the cortical activity of the PFC or of the M1 during walking conditions, before and after the procedure (Morishita et al., 2016).

In MS field, most fNIRS reports are cross-sectional studies that investigated cortical activity mostly in frontal areas, employing different tasks, ranging from simple dual-task conditions, static motor protocols, and working memory (WM) tasks (for extensive review of literature see Bonilauri et al., 2020).

1.2.1.2 fNIRS application in psychiatry

In the past two decades, fNIRS has also been increasingly used to conduct functional activation studies in different psychiatric disorders (Ehlis et al., 2014), most prominently schizophrenic illnesses (Kumar et al., 2017), affective and anxiety disorders (Nishimura et al., 2009) as well as addiction and personality disorder, especially borderline personality (Ruocco et al., 2010).

Studies cover a wide range of objectives, including studies on the phenomenological characterization of psychiatric disorders (investigating differences in HRF between various groups of psychiatric patients and healthy control samples), descriptions of life-time developmental aspects, treatment effects of specific drugs or non-pharmacological interventions, and genetic influences on neuroimaging data (even if a few studies

focused on psychiatric cohort, particularly schizophrenic, patients)(Takizawa et al., 2009). From a methodological point of view, most fNIRS studies in psychiatric settings have been conducted on prefrontal activation, with the VFT being the most popular paradigm (Dieler et al., 2012). Overall, fNIRS studies in schizophrenia detected abnormal (lower) brain activation patterns in patients compared to controls, particularly within PFC, during various neurocognitive tasks such as random number generation, Tower of Hanoi, Stroop Test, VFT and Go/Nogo tasks, thus replicating the general finding of cerebral hypofrontality revealed by other neuroimaging methods. Additionally, prefrontal hemodynamic data were partly found to be impacted by clinical characteristics (i.e., age at onset), medication effects (Watanabe & Kato, 2004), and psychopathological symptom scores (Ehlis et al., 2014). Moreover, recent NIRS studies have demonstrated some promising data of altered resting state in the prefrontal cortex of patients with schizophrenia (for a detailed review see Yanagi et al., 2021).

Similarly, frontal areas were usually the ROI of most fNIRS studies in affective disorders such as, unipolar and bipolar disorder. Here, a reduced frontal lobe function has generally been confirmed during WM and VFT performance, even in remitted/euthymic stages of the disease (Kopf et al., 2023).

fNIRS studies on anxiety disorders have so far focused on panic disorder and post-traumatic stress disorder (PTSD), highlighting atypical pattern, again, of PFC activation during confrontation with anxiety-relevant material (Akiyoshi et al., 2003).

Finally, fNIRS seems to be a promising tool in practical psychiatric settings involving both diagnostics and the complementary treatment of psychological disorders using neurofeedback system, such as, for example, its application in social anxiety (Kimmig et al., 2019).

1.2.2 fNIRS studies in ageing

According to a recent systematic review of the literature (Yeung & Chan, 2021), more than hundred fNIRS papers have examined changes in the oxygenation of cerebral blood in older adults, suggesting that healthy aging is a growing area of scientific interest. Altogether, these studies suggest that aging is associated with changes in the activation and connectivity patterns of the brain, especially the PFC, both in resting state and task-related condition.

The majority of fNIRS reports using resting-state recordings show that, compared to younger adults, older ones have lower basal PFC oxygenation. High reproducibility of the results was observed between research groups (Harada et al., 2007; Tan et al.,

2016). Additionally, some studies have shown that aging is associated with modification in interhemispheric connectivity, in particular reduced connectivity between the left and right PFC, and increased connectivity between the PFC and sensorimotor cortex (i.e., increased influence from the motor cortex to the PFC). Apart from PFC oxygenation levels and connectivity between brain regions, other researches have focused on age-related change in the coupling of PFC activity and arterial blood pressure and the age-related attenuation of neurovascular coupling due to increased vessel stiffness and impaired myogenic response (Mukli et al., 2018). These findings are consistent with PET findings of age-related decline in regional blood flow and glucose metabolism in the frontal lobe (Kalpouzos et al., 2009). Various mechanisms are thought to underlie these age-related changes in frontal oxygenation and brain networks, ranging from deteriorated neurovascular coupling to age-related deterioration of white matter tract affecting PFC functioning and laying to compensatory functional connectivity to other cortical regions.

Regarding task-evoked paradigms, in particular involving sensorimotor functioning, fNIRS studies suggest that aging is associated with hyperactivation of the sensory cortex during active perceptual tasks (Lin et al., 2017; Ward et al., 2018), while being hypoactivated during passive stimulation tasks (Ward et al., 2015). Hyperactivation in the specialized sensory cortex may reflect the presence of compensatory mechanisms, such as an increased reliance on sensory reweighting, under perceptually challenging conditions. Similarly, the fNIRS literature suggests that aging is associated with frontal hyperactivation during simple motor tasks (Hawkins et al., 2018), but hypoactivation during complex motor tasks, such as dual-task walking and driving (Harada et al., 2007). Moreover, fNIRS findings suggest that age related frontal hyperactivation is indicative of compensatory activity across multiple cognitive operations (Agbangla et al., 2017), including verbal domain, such as word retrieval, working memory and semantic processing (Heinzel et al., 2015; Vermeij et al., 2012), task-switching and multitasking (Laguë-Beauvais et al., 2013), motor inhibition and interference control (Kawai et al., 2020). Taken together, this evidence would indicate that older adults recruit non-specialized regions to compensate for their functional decline in specialized regions. Importantly, the fNIRS literature is quite consistent with fMRI metanalytic studies reporting age-related frontal hyperactivation across multiple cognitive domains (Li et al., 2015).

Although aging is associated with changes in resting cerebral hemodynamics in general, due to a reduced efficiency of cerebral autoregulation process in elderliness, there is

considerable inter subject variability of hemodynamic response (Suhr & Chelberg, 2013), suggesting some other factors to take into account. Notably, some studies have shown that physical exercise and sleep quality are two lifestyle habits that exert a modulating effect over resting cerebral oxygenation among healthy older adults; in particular, fNIRS recordings on elderly subjects suggested that poor sleep reduces brain phase synchronization, which may contribute to the diminished cognitive functions among the sample population (Bu et al., 2018).

Although fNIRS is a promising tool to study healthy aging, it has some limitations and potential pitfalls, in particular a limited depth of light penetration that prevent to measure activity in deep brain structures, such as the limbic system and subcortical regions. In addition, fNIRS measures neural activity without providing anatomical information about the brain regions being studied, raising possible critical issue in population with putative neurovascular comorbidity such as elderly one. Thus, the relationship between age-related changes in brain structure and function cannot be determined solely using this method.

Despite these methodological issues, the growing application of fNIRS to elderly research will help to improve neurobiological models of aging, inspire interventions that might be used to enhance the brain function of older people, and identify biomarkers of aging that can be used to differentiate healthy and pathological aging (Yeung & Chan, 2020). In particular, some fNIRS studies have already focused on MCI and dementia like AD, in order to replicate evidence from other neuroimaging technique (Yoon et al., 2023) and tailor rehabilitative interventions (Viola et al., 2014).

1.2.3 fNIRS studies in neonates, infants and children

The past decade has seen a dramatic increase of neuroimaging studies of infancy, shedding light on typical and atypical neurodevelopment since the first months of life (Azhari aet al., 2020).

Neuroimaging studies in young subjects have been limited by challenges posed by this particular cohort in experimental settings, including practical and procedural difficulties such as sustaining the attention and the compliance of subjects, artefact movements diminishing the quality of data, and the need for friendly procedures (Raschle et al., 2012). Despite of these difficulties, application of functional neuroimaging research in infancy and childhood has rapidly increased and given precious insight into brain plasticity. In this respect, within several functional techniques available (i.e., EEG, MEG

and fMRI), fNIRS stands out for his non-invasiveness and feasibility in very young subjects (even if with high dropout rates compared with adults). In the last years, much time and many resources have been applied to technical improvement to maximize the effectiveness of NIRS as a tool for infant research, i.e., designing increasingly sophisticated array of channels for NIRS probes and headgear in order to be comfortable and accurate. As previously reported, conducting a fNIRS study in younger population needs specific methodological foresights, linked to apparatus and experimental design used. Notably, mostly before 2 years of age when brain is rapidly growing, optimal emitter-probe distance need to be *a priori* accurately discussed according to age of the subject and ROIs to study. Moreover, non-canonical HDR can be often recorded in this range of age, requiring a more complex interpretation of results.

As for adults, in pediatric population the fields of application of fNIRS are wide, ranging from language and learning abilities, to motor system, to cognitive and social domains, both in typical and atypical population. Growing interest in the field is rising from longitudinal studies, that propose causal relationships in neurodevelopment rather than cross-sectional ones primarily indicate developmental patterns devoid of causal inferences. According to a recent review (Su et al., 2023), to date there are almost 84 fNIRS longitudinal studies looking at developmental trajectories in pediatric population, the majority of these focused specifically on infant and toddler populations, while the others focused on the school age children and adolescents or transition to adulthood. Most of these studies only included Typically Developing (TD) infants/children/adults, Overall, fNIRS studies on infants and children show a general trend of age-related increase in network integration and segregation, interhemispheric connectivity, leftward asymmetry, and differences in phase oscillation during resting-state. Moreover, a developmental trend of more localized and differentiated activation when processing visual, auditory, and tactile information has been noted, suggesting more mature and specialized sensory networks. Later in life, children switched from recruiting bilateral auditory to a left-lateralized language circuit when processing social auditory and language information and showed increased prefrontal activation during executive functioning tasks.

Findings of fNIRS studies in typically developing infants and children are crucial for interpreting the results of studies in atypically developing populations. In particular, the studies of several social (i.e., joint attention, face perception, language acquisition) and cognitive domains (i.e., executive functioning such as inhibition, cognitive shifting, and working memory) have been fundamental to enucleate core deficits of

neurodevelopmental disorders such as ASD and ADHD, respectively. Moreover, ongoing research into understanding the hemodynamic changes that occur in the sensory systems in the infant brain is continuously growing (Aslin, 2012) and rising interest in the use fNIRS to evaluate (and potentially predict) preterm motor development (de Oliveira et al., 2019). Notably, NIRS has also been applied to monitor regional cerebral oxygenation (cRSO2) in healthy babies (Tina et al., 2009) as well as in Neonatal Intensive Care Unit (NICU) for newborns in critical conditions (Pichler et al., 2014), claiming then the usefulness of this tool also as an early potential predictor of outcome in perinatal asphyxia (Nicklin et al., 2003) or in prematurity (Chock et al., 2020).
Starting from these considerations, the next paragraphs will focus on fNIRS application to study typical development in several brain domains ranging from primary sensory areas and sensorimotor cortex ("low-level" cortical processing) to more complex cognitive process such as social cognition, executive functioning and language, while in the section "Application of fNIRS in neurodevelopmental disorders" fNIRS application in atypical neurodevelopment will be extensively described.

1.2.3.1 Primary sensory areas and sensorimotor cortex studied with fNIRS

FNIRS has been proven reliable for investigation of primary cortices involved in sensory perception and motor execution both in adults and in children. Often simultaneous fNIRS-EEG experiment has been conducted in this field in order to explore the precise relationship between changes in neural activity and hemoglobin concentrations (Chen et al., 2015).
Regarding sensory processing, several studies focused on the characteristics of such "low-level" cortical activation, mostly in visual and auditory perception (for the latter see review of van de Rijt et al., 2018). Altogether these findings demonstrate area specificity of fNIRS response and stimulus selectivity using both visual task (Takahashi et al., 2000) and auditory ones (Chen et al., 2015). Moreover, a stimulus-dependent modulation of the fNIRS signal could be observed for luminance level in the visual area, as well as for loudness or intensity variability in the auditory area, suggesting sensitivity of fNIRS response to trigger's characteristics (Chen et al., 2015; Bauernfeind et al., 2018). Similar findings arise from studies on developmental age, outlining a detectable specific occipital response to simple visual stimulus (like checkerboard pattern) starting from 3 months-old infants (Watanabe et al., 2008). Further exploration on early visual processing came from the study of photostimulation of infants during natural sleep, revealing non-canonical HDR with decreasing of OHb and THb and increasing of DHb

(Kusaka et al., 2004). Auditory processing in infants has been typically studied using more complex stimuli, such as speech-like sounds, however demonstrating early specialization of temporal brain areas (Bortfeld et al., 2007; Gervain et al., 2008). To date, sporadic reports describe the use of pure tone stimuli in infants but in the context of oddball experimental paradigm, thus, to evaluate longitudinally habituation and novelty detection rather than auditory response itself (Katus et al., 2023). Moreover, isolated studies used auditory-evoked response to monitor broad cerebral oxygenation changes in newborns with hypoxic-ischemic encephalopathy (Chen et al., 2002). Furthermore, brain activation in response in the frame of learning experimental paradigm has been widely studied in infants (Emberson et al., 2015), supporting the hypothesis of a predictive coding in sensory processing (Friston, 2005). Interestingly, recent studies found that prematurity, altering top-down prediction network, might also cause atypical response in sensory perception, mostly of visual stimuli (Boldin et al., 2018).

Regarding tactile sensation, which is one of the earliest developing sensory systems, fNIRS has been applied since first days of life, highlighting that in early newborn period the tactile stimuli induce broader areas of brain activation than the other types of stimuli (Shibata et al., 2012). These results suggest that there are differences in newborns' reactions to various types of sensory stimuli, which may reflect the importance of tactile sensation in the early newborn period. Interestingly, several studies addressed also the issue of nociception experience in newborn suggesting the promising role of a such non-invasive technique like fNIRS in NICU to early detect pain in critically ill infants (Ranger et al., 2011)

One domain in which there is very little research in infancy and childhood is action observation and/or execution in either typical or atypical development, while more evidence comes from studies in adults (for a recent review on the field see Condy et al., 2021). The neural mechanism underlying these behaviors has recently gained popularity for its potential connection to the human mirror neuron system (MNS), a neural network that presents similar activation while an individual performs a goal-directed action and while he observes another performing it. The human MNS is comprised of the sensorimotor cortex, inferior frontal gyrus (IFG), and inferior parietal lobe (IPL) (Buccino, et al., 2001). Because of the proximity to the scalp of these three regions, the use of fNIRS presents a unique opportunity to measure the early development of the MNS and its potential role in atypical development. Indeed, many have hypothesized a link between action understanding and the development of more

complex social cognition, such as joint attention, imitation, theory of mind, and empathy.

To date, in infancy evidence using fNIRS focused mostly on action observation: in particular interesting findings came from the study of Lloyd-Fox et al. (2011) and Grossmann et al. (2013). In the first study, videos of a woman moving her eyes, opening and closing her mouth, and making a fist with her right hand was presented to 5-month-old infants (Lloyd-Fox et al., 2011). The biological movement conditions were contrasted with a baseline condition of moving toys. The study revealed an increase in OxyHb during the stimulus condition, the earliest demonstration that observation of biological motion activates motor regions in human infants. In the second study, four videos of an individual performing dance was presented to 4-month-old infants (Grossmann et al. 2013). In two of the videos, there was a human performing a natural dance or robotic movements and in the other two videos a Lego man was animated to match the two dances performed by the human. Contrary to the results reported by Lloyd-Fox et al. (2011), there was no clear increase in OHb in channels over the IFG region to the observation of human actions. These two studies represent first attempts to measure MNS activation to biological motion with fNIRS in infants; however, no study has measured activity in these regions during action execution. The functional activity of the sensorimotor cortex, IFG, and IPL represent important regions for fNIRS researchers to focus future efforts.

Altogether these findings demonstrate that fNIRS could be a reliable and non-invasive candidate tool to explore brain activation in response to simple trigger since early developmental age, theoretically suitable also for more complex stimuli and cognitive domains.

1.2.3.2 The study of language development with fNIRS

The availability of a non-invasive method to investigate neuronal networks underlying language processing is particularly crucial due the fast pace of learning of this complex cognitive function during the first years of life. The applicability of the methodology in infants opens the possibility to assume a life-time perspective and compare results in adults with language acquisition processes in preverbal infants.

To date, there is a wide range of studies focused on auditory language processing and acquisition, both in developmental age and adulthood (see review of Quaresima et al., 2012), variably targeted to efficiency of word and sentence elaboration (involving phonological and prosodic features as well as semantic and syntactic information) (Rossi

et al., 2012), but also hemispheric lateralization of auditory processing and its evolution during development (Gervain et al., 2011).

From a historical point of view, in 1998, the activation of the Broca's area during language processing was demonstrated for the first time by utilizing the first commercial fNIRS system (the 24-channel Hitachi ETG-100) (Watanabe et al., 1998). Thereafter, almost all the published data have been obtained employing multi-channel systems (up to 128 channels). A robust cortical activation has been observed in the brain's classic language areas in children/adults as well as newborns using instrumentations of different complexity. A wide variety of sounds has been used as auditory stimulations on infants (for instance, artificial sounds, music and speech) for addressing some important issues such as the development of multimodal perception, the mechanisms underlying the acquisition of auditory and speech patterns, the brain mechanisms underlying language learning, the development of hemispheric lateralization of speech and music responses (due to the overlap between social and language studies in this field, principal findings are briefly described in next paragraph).

The processing of semantic information in monolingual and bilingual language contexts was extensively investigated too using fNIRS, counting to date almost twenty studies in literature.

Moreover, the method may be especially helpful in patients with cochlear implant (CI) as well as in adults suffering from aphasia after stroke event. Indeed, outcomes following cochlear implantation vary widely for both adults and children looking at behavioral tests that, unfortunately, can be unreliable, particularly for younger subjects. Thus, lots of scientific effort has been done to asses with more objective measure like fNIRS the effect of CI on language network re-organization (Harrison et al., 2021).

1.2.3.3 FNIRS in social neuroscience

A range of social perception and interaction studies in children have been implemented with fNIRS, particularly focusing on identifying what regions of the developing brain preferentially respond to social vs. non-social (or less social) stimuli. Some of these studies are similar to typical fMRI studies, examining face perception, emotion perception, and theory of mind in both child and adult populations (i.e., emotion recognition in mothers and infants testified by activation of PFC when seeing videos of the other smiling) (Minagawa-Kawai et al., 2009). But, building on the participant-friendly nature of fNIRS, a larger number of studies use this method to explore the development of cognition and social cognition have been carried out in infants, children,

and individuals with developmental disorders, even with more naturalistic and interactive experimental settings. Such work tracks the development of body perception (Grossmann et la., 2013) response to direct gaze (Lloyd-Fox et al., 2015), responses to speech (Bortfeld et al., 2007) and many other tasks.

The majority of fNIRS studies of infant social development have focused on the visual domain, overcoming the difficulty of collecting fMRI data with awake infants. To date, fNIRS studies have examined brain responses to dynamic visual social stimuli such as human movement, faces, facial emotion, and eye gaze (for extensive review of results see McDonald et al., 2018). From the first days of life, areas of the right and left posterior temporal cortex selectively respond to dynamic face stimuli (i.e., videos of an adult playing peek-a-boo; Farroni et al., 2013). At 5 months, infants continue to show increased bilateral activation to videos of social movement in the posterior temporal cortex, with a right hemisphere dominance of response at this age and different topological patterns of brain activation in response to specific socially-relevant body movements such as eye movement, hand movement, mouth movements (Lloyd-Fox et al., 2011). In addition to human movements, images of faces have been used to examine infant brain responses to faces and facial expressions. fNIRS studies focused on infant brain responses to canonical vs. scrambled or inverted faces suggest preferential activation of the right temporal area in social perception by mid-way through the first year of life (Honda et al., 2010). fNIRS has also been used to study infant brain responses to facial emotional expressions, highlighting from 7-months of age, different patterns of brain responses to happy vs angry faces, with regard to both the localization and timing of activity (Nakato et al., 2011). Moreover, Grossmann and colleagues have conducted a series of fNIRS experiments examining infant brain responses to aspects of joint attention, finding that already by 4–5 months of age infants distinguish between mutual vs. averted gaze conditions in areas of the frontal and temporal cortices and this pattern modifying longitudinally (Grossmann et al., 2008).

There have been also a growing number of fNIRS studies focused on examining infant brain responses to pre-recorded auditory social stimuli, particularly investigating brain responses to human vocalizations, differences in speech prosody (including infant-directed speech), and socially-relevant speech (i.e., response to name) (for extensive review of results see McDonald et al., 2018). Compared to previous fMRI studies, fNIRS in this field shows methodological advantages including the relative silence and

increased accessibility of fNIRS systems, and, as noted above, the ability to assess brain responses while infants are awake.

Numerous studies have contrasted infant brain responses to human vocalizations (e.g., laughing, coughing) vs. non-social sounds (e.g., rattle, running water), consistently finding that infants begin responding preferentially to social vs. non-social sounds in the second half of the first year of life as testified by increased activation to social stimuli in the left and right temporal cortex (Grossmann et al. 2010a). Differential responses to variations in prosody have also been examined in infants using fNIRS, showing that at 7 months of age infants further differentiate cortical response emotional tone (Grossmann et al., 2010a). In addition to studying basic vocalizations and prosody, fNIRS has also been used to study the neural underpinnings of infant response to name, that is considered an important developmental milestone in social development. At 5 months, an age at which clear behavioral indicators of response to name are often not evident, infants have shown increased activation in response to their own (vs. another) name in an fNIRS channel in the left dorsal PFC (Grossmann et al., 2010b).

Overall, these studies suggest that areas that are involved in social information processing during adulthood appear to rapidly become sensitive to social stimuli within the first year of life, including the posterior superior temporal sulcus (STS)-temporoparietal junction (TPJ) area region and areas of the PFC. Moreover, typically developing infants begin to show preferential responses to multi-modal social stimuli in areas of the temporal and frontal cortices within the first 6 months of life. Interestingly, infants at elevated risk for social deficits demonstrate differential responses to pre-recorded social stimuli during the first year of life, prior to observable behavioral differences (Jones et al., 2014).

Finally, as previously mentioned, one of the most exciting and rapidly growing areas of fNIRS in social neuroscience is hyperscanning, where signals are recorded from two or more participants simultaneously. Notably, hyperscanning studies of mother-child interaction allow to demonstrate pattern of typical and atypical, highlighting the harmful role of parenting stress; for example, the study of Azhari and colleagues has shown that greater parenting stress is associated with less brain-to-brain synchrony in the medial left cluster of the prefrontal cortex when mother and child engage in a typical dyadic task of watching animation videos together (Azhari et al., 2019). Sporadic reports on fNIRS hyperscanning come also from NDDs field and in particular this technique seems to be particularly promising for the study of neurobiological underpinnings of ASD. For example, Wang and colleagues have studied level of interpersonal neural

synchronization in the frontal cortex within ASD children and their parents in cooperative interactions or during non-interactive behaviors: interestingly, this neural synchronization was modulated by the children's autism symptoms, which also covaried with their cooperation task performance (Wang et al., 2020).

1.2.3.4 Executive functions explored with fNIRS

Executive functions (EF) refer to the higher-order cognitive control process for the attainment of a specific goal. There are several subcomponents of EF, such as cognitive shifting, working memory and inhibition (Lehto et la., 2003). Extensive neuroimaging research in adults has revealed that the lateral prefrontal cortex plays an important role in EF. Developmental studies have reported behavioral evidence showing that EF changes significantly during preschool years. Similarly, there is some anatomical evidence obtained with MRI studies that the prefrontal cortex develops during preschool years (Gogtay et al., 2004) but little functional neuroimaging data have demonstrated the functional development of these cortical areas in young children. Interestingly, fNIRS has shown that prefrontal activation is developmentally correlated with EF in young children with a pattern of response that is age-specific.

Many brain imaging studies using fMRI have shown that adult participants recruit inferior and dorsolateral prefrontal regions during cognitive shifting tasks, such as the Wisconsin Card Sorting Test (WCST) (Monchi et al., 2001). In the WCTS participants are asked to sort cards depicting geometric features (such as shape, color, and number) according to not-explicit rules that are detected through feedback progressively given (and changed) by an experimenter. In preschoolers, developmental studies have shown that 3-year-old children often perseverate to previous mental sets, whereas 5-year-old children do not. fNIRS has given direct supporting evidence that maturation of the prefrontal cortex, particularly inferior prefrontal activation, plays an essential role in the development of successful shifting in young children (Moriguchi & Hiraki, 2009). Regarding WM task, evidence from older children and adults has consistently shown that regions in the prefrontal cortex, such as the dorsolateral prefrontal cortex, play an important role in visuospatial working memory (Braver et al., 1997). Overall, fNIRS research has demonstrated that children activate the right prefrontal regions during visuospatial working memory tasks, similarly to adults. Early developmental changes in WM, however, were more controversial with some studies showing stronger activation in older children (Buss et al., 2014) whereas other reporting weaker activation and less right-lateralized responses in older children (Tsujii et al. 2009). Instead, few NIRS

studies have been conducted on the development of inhibitory control in young children. On this topic, interesting finding came out from studies comparing preschoolers (4- to 6-year-old children) and adults performing a Go/NoGo task, where participants were asked to respond to targets by pressing a button (Go trials) and to avoid making a response to non-targets (NoGo trials) (Mehnert et al. 2013). fNIRS results showed that adults activate right frontal and parietal regions during NoGo trials compared to Go trials, whereas children's right frontal and parietal activation was high in both Go and NoGo trials.

Collectively the available results highlighted that prefrontal activation may change developmentally across different tasks (e.g., working memory and cognitive shifting tasks), suggesting the possibility that the prefrontal regions may be generally activated across different tasks in preschoolers, but may become more localized to specific regions in older children. Moreover, how the development of prefrontal function may be related to other aspects of socio-cognitive skills, such as theory of mind, communicative skills, and emotional regulation, is still an open question. Interestingly, recent research has tried to fill this gap examining the relationship between prefrontal activation and emotion regulation, showing that dorsolateral PFC activation was associated with cognitive flexibility but also irritability in children, thus, suggesting the entwined nature of cognitive and emotional neurodevelopment since first years of life (Li et al., 2017).

1.3 Application of fNIRS in Neurodevelopmental Disorders

Understanding the neurodevelopmental trajectories of infants and children is essential for the early identification of neurodevelopmental disorders, elucidating the neural mechanisms underlying the disorders, and predicting developmental outcomes.

Although the growing number of studies using fNIRS so far, unfortunately its role in diagnostic or rehabilitative pipeline (for the early identification of developmental disorders and in tracking the effects of interventions) is not standardized yet due to a lack of synthesized evidence.

A brief summary of fNIRS evidence in principal NDDs such as ASD, ADHD and Developmental Dyslexia is respectively given in next paragraphs. Compared to typically developing (TD) peers, children with aforementioned developmental disorders, demonstrate atypical neurodevelopmental trajectories during resting-state and when performing executive functioning tasks. In order to investigate the application of fNIRS

to NDDs in younger population, a systematic review of literature has been recently performed focusing specifically preschoolers, finding as principal application of technique in this subgroup the assessment of ASD children or high-risk infants (Conti et al., 2022). Regarding other NDDs, fNIRS studies on rare diseases with Intellectual Disability (ID) is only anecdotal yet (Bembich et al., 2021).

1.3.1 Language impairment and Learning Disabilities

As mentioned before, a notable bulk of fNIRS literature is dedicated to language research, even if mainly in typical developed children. Indeed, fNIRS was rarely used to investigate neural underpinnings of language disorder such as developmental stuttering, a complex, multifactorial condition characterized by frequent disruption of the fluent flow of speech. In particular, the extent of laterality (left versus right cerebral dominance) in brain function for phonological and prosodic contrast tasks was assessed with fNIRS in adults, school-aged children, and preschool-aged children who stutter (Sato et al., 2011). The researchers found that age-matched nonstuttering speakers consistently exhibited greater left than right laterality of brain response when listening to auditory stimuli differing in phoneme versus prosody. In contrast, not even one subject among the stuttering group exhibited leftward laterality for the phoneme versus prosodic contrasts. Relatively more evidence comes from learning disabilities, in particular developmental dyslexia (Marks et al., 2022). Developmental dyslexia is characterized by a deficit of phonological awareness whose origin is related to atypical neural processing of speech streams. Thus, fNIRS has been applied to study cortical networks derived from low-level auditory processing of nonspeech stimuli related to speech units such as stress, syllables or phonemes, suggesting the presence of discrepancies in the topological organizations of functional brain networks and their dynamics that differentiate between control and dyslexic subjects (Gallego-Molina et al., 2023). Moreover, other studies support the presence of atypical cortical activation in the left prefrontal cortex in dyslexic subjects indicating a deficiency in working memory (Zhu et al., 2012).

1.3.2 Autism Spectrum Disorder (ASD)

Autism spectrum disorder (ASD) is a complex neurodevelopmental condition characterized by early-onset and persistent challenges in social interactions, impaired communication, restricted interests, repetitive behavior, and alterations of sensory

processing (Hyman et al, 2020). FNIRS is increasingly being used to investigate the functional development of the brain in ASD. A recent review of Zhang and colleagues (Zhang et al, 2019) found thirty empirical studies, published in the past decade, that used fNIRS in individuals with ASD or in infants with a high risk of developing ASD. These studies investigated either brain activation using multiple tasks (e.g., social task such as face processing and joint attention, but also EF such as working memory) or functional organization under a resting-state condition in ASD. The majority of these studies reported atypical brain activation in the prefrontal cortex, inferior frontal gyrus, middle and superior temporal gyrus; main findings are reported as follows (for a more extensive description see the review of Zhang et al., 2022). The majority of fNIRS studies have been conducted in schooler ASD children with normal intellectual functioning or in adult subjects. Many studies focused on facial processing, finding a decreased response in individuals with ASD compared to peers with typically development in the IFG during self-face recognition (Kita et al., 2011), as well as in the PFC when processing fearful faces (Nakadoi et al., 2012). With regard to social-cognitive tasks including joint attention and imitation, the results of fNIRS studies showed abnormal activity patterns in children with ASD in the frontal cortex, such as lower activation in the PFC when following eye gazes of others (Zhu et al., 2015) as well as reduced activation in the IFG and decreased left lateralization in the rostral PFC for imitation tasks (Tamura et al., 2012). Moreover, almost ten fNIRS studies have investigated executive functions in ASD, mostly working memory and inhibition domains. These studies observed an atypical activation in the PFC during letter-fluency tasks (Kuwabara et al., 2006), working memory tasks (Yanagisawa et al., 2016) and task switching/ inhibition (Funabiki et al., 2012). However, ASD participants showed task-specific heterogeneity of the PFC pattern of response. It is worth noting that fNIRS signal has proven its usefulness as an auxiliary tool for the differential diagnosis of ASD and ADHD starting from divergent HDR in left ventrolateral prefrontal activation during inhibitory control between these two cohorts (Ishii-Takahashi et al., 2013).

Interestingly, fNIRS has been used as an early marker of atypical neurodevelopment, even prior to the full clinical manifestation. In particular, fNIRS has been extensively used to study infant population at high risk (HR) for autism like siblings. To date, five papers investigated social perception and three others assessed possible alterations of language processing in the HR group (for a recent review see Conti et al., 2022). In particular, the pioneering study of Lloyd-Fox group used fNIRS to identify differences in neural responses to visual and auditory social stimuli since 4-month-old infants at risk

for autism, indicating its potential for early diagnosis (Lloyd-Fox et al., 2013; Lloyd-Fox et al., 2018).

Moreover, regarding resting-state fNIRS recording, some studies revealed altered functional connectivity, suggesting an inefficient information transfer between brain regions in ASD. Taken together, fNIRS studies indicate that children with ASD show an initial outgrowth followed by a later decrease in resting-state functional connectivity (for a recent review see Zhang et al., 2022), consistently with evidence from other neuroimaging technique. Indeed, although fNIRS results are sometimes inconsistent between research groups, this developmental pattern has also been reported in EEG and fMRI studies in children with ASD, which may be explained by initial short-range overconnectivity and later long-range underconnectivity (Uddin et al., 2013).

1.3.3 Attention Deficit / Hyperactivity Disorder (ADHD)

ADHD patients have deficits in executive functions, the higher-level cognitive functions necessary for mature adult goal-directed behaviors, that are mediated by late developing fronto-striato-parietal and fronto-cerebellar networks (Rubia, 2013). The most consistent deficits are in so-called ‘‘cool’’ EF such as motor response inhibition, working memory, sustained attention, response variability and cognitive switching as well as in temporal processing (i.e., motor timing, time estimation and temporal foresight), with most consistent deficits in time discrimination and estimation tasks (Rubia, 2018). However, impairment has also been found in other EF domains such as motivation control and reward-related decision making (i.e., temporal discounting and gambling tasks), even if with more inconsistent findings. fNIRS has been used as valuable tool to record activation mainly of frontal circuits, in order to study neurobiologic underpinnings of ADHD or to monitor response to specific pharmacological therapy (methylphenidate or atomoxetine).

To date, more than 50 studies have been published on this topic, suggesting that children and adolescents with ADHD show peculiar cortical activation both during neurological and emotional tasks consisting of a lower prefrontal cortex activation in patients compared to typically developmental peers (for recent review on this topic see Mauri et al., 2018). Among the executive functions, impaired inhibition is frequently observed in children with ADHD in fNIRS studies while performing typical behavioral test such as stroop task, reverse stroop task or Go/No-Go task. The group of Negoro (Negoro et al., 2010) reported that children with ADHD showed significantly smaller changes in oxy-hemoglobin than TD children in the inferior lateral PFC bilaterally during

the Stroop color-word task. The group of Yasumura. (Yasumura et al., 2014) reported that inattention severity in the ADHD group was negatively correlated with right lateral PFC activity during the reverse stroop task. The group of Kaga (Kaga et al., 2020) reported that children with ADHD showed a greater decrease in hemodynamic activity in the right PFC than those in the TD group using fNIRS during the Go/No-Go task. Although the studies differed slightly in terms of methods, the hemodynamic activity of the right PFC in the ADHD group was consistently lower than in control group.

Moreover, twelve articles describe longitudinally the effects of specific pharmacotherapy in ADHD samples. The majority of the studies revealed an increasing of oxygenated hemoglobin concentrations in the prefrontal cortex following Methylphenidate or Atomoxetine treatment, finding often an increased HDR in the right dorsolateral and ventrolateral PFC regions (for an extensive review on this topic see Grazioli et al., 2019). Thus, both in cross-sectional and longitudinal fNIRS studies on ADHD an interesting pattern of lateralization of HDR has been reported (Doi et Shinoara., 2017), supporting the right-lateralized atypicality in children with ADHD previously described with behavioral assessment and other neuroimaging techniques (Stefanatos & Wasserstein, 2001; Valera et al., 2007).

More recently, researchers have begun to investigate the feasibility of fNIRS-neurofeedback training protocols. Neurofeedback is a form of biofeedback in which subjects respond to a display of their own brainwaves or other neural activity of the nervous system, typically obtained with EEG or fMRI signal. fNIRS-neurofeedback effectiveness in changing behavior in healthy and pathological populations (such as ADHD) remain still difficult to interpret because studies are methodologically heterogeneous and large randomized controlled trials are still lacking (for an extensive review see Kohl et al., 2020). Nevertheless, due to the advantages of practicability and relatively low cost, fNIRS-neurofeedback might provide a suitable and powerful alternative to EEG and fMRI neurofeedback in future research.

To conclude, to date fNIRS has robustly proved its validity in several neuroscience fields and age, ranging from application in very young to elder subjects. Notably, fNIRS is a promising tool for studies focused on infancy and childhood, thanks to non-invasiveness and flexibility of technique. Indeed, there is a growing interest to track developmental trajectories in typical and, mostly, atypical neurodevelopment and fNIRS might represent a potential and reliable biomarkers of brain function in the next years.

2 Aim of the thesis

Functional near-infrared spectroscopy (fNIRS) has widely demonstrated its usefulness to study both typical and atypical brain development (Vanderwert & Nelson, 2014). However, fNIRS application as a biomarker of brain function in NDDs and, mostly, in rare diseases, remains sparse yet.

In this field, the increasing availability of innovative disease-modifying interventions leads to a concomitant urgent need of identification and validation of practical and reliable outcome measures and biomarkers for future clinical trial, including drug evaluation studies (Lee et al., 2018). Theoretically, clinical readouts should test a broad range of ability and quantify core defects, overcome cooperation/variable performance problems (leading to limited standardization and floor effects), correlate with quality of life, and assure high reproducibility of results. Unfortunately, these desirable outcome measures are very difficult to obtain in NDDs population, with clinical readouts being highly prone to subjective bias. Similarly, an ideal biomarker should be objective, reliable and sensitive in order to optimize clinical assessment and provide unbiased endpoints of treatment response as well as predictors of outcome.

In this scenario, neuroimaging biomarkers and in particular a non-invasive technique such as fNIRS might cover a crucial role. A growing number of studies pointed to visual impairments across multiple NDDs and to visually evoked responses as functional biomarker for the study of the severity and progression of such disorders (LeBlanc et al., 201; Saby et al., 2021; Saby et al., 2022). Accordingly, preclinical studies using intrinsic optical signal (IOS) imaging (Zepeda et al., 2004) demonstrated that the assessment of cerebral oxygen consumption in response to visual tasks can effectively discriminate between healthy and pathological brain circuits in several NDDs models (de Freitas Dotto et al., 2014; Mazziotti et al., 2017; Felgerolle et al., 2019; Mazziotti et al., 2020). Interestingly, both IOS imaging and fNIRS are optical techniques providing an indirect measure of neuronal activity and relying on the same physiological sources, i.e., the changes of hemoglobin species and local blood flow in in active brain regions.

Thus, hypothesizing that HDR represents a sensitive readout for quantifying functional alterations of neural circuits, the aim of this research project was to assess the feasibility, validity and reliability of fNIRS visual recordings in several cohorts of NDDs, with regard, especially, on under investigated clinical population including rare genetic disease.

To this purpose, a novel visual-evoked fNIRS paradigm has been designed to increase the entertaining value of hemodynamic measurements (HDR) in the occipital cortex. A

first standardization of this novel experimental paradigm has been proved in normative adult population. In order to overcome well-known methodological issues in young population, some implementations have been done to optimize compliance of children, demonstrating technique feasibility and consistency of HDR metrics compared to adults. Reliability and reproducibility of the measure has been longitudinally assessed as well, evaluating test/retest consistency at three different timepoints.

Then, the feasibility and validity of visual HDR in atypical neurodevelopment were assessed in two pilot cross-sectional studies. The first one was conducted in a cohort of preschooler autistic female (fASD) in order to explore a novel non-invasive tool supporting diagnostic pipeline. Indeed, differences in clinical presentation typically make diagnosis more challenging in females than in males with significant negative implications for the treatment and the quality of life for women with autism. In addition this activity would provide desirable novel insights about possible neurobiological underpinnings of fASD. A second cross-sectional study aimed to explore the potential application of visual HDR in Creatine Transporter Deficiency (CTD) syndrome, a rare metabolic disease causing a lack of creatine in the brain and, thus, a misleading energy use in neuron. The diagnostic pathway of CTD is well established but there is a pressing need of reliable readouts for testing the efficacy of potential drugs. Thus, starting from encouraging data in the preclinical mouse model (Mazziotti et al., 2020), fNIRS signals have been used to explore the validity of this metric to discriminate atypical brain activation in CTD patients compared to controls.

3 Materials and methods

3.1 Apparatus and montages

Our fNIRS apparatus has been optimized to measure changes in total Hb (THb) concentration and relative oxygenation levels (OHb and DHb) in the occipital cortex during a visual task.

Technical equipment consists of a continuous-wave NIRS system (NIRSport 8×8, NIRx Medical Technologies LLC, Berlin, Germany). Our NIRSport system includes eight red light-sources operating at 760 and 850 nm, and eight detectors that can be placed into a textile EEG cap (EASYCAP, Herrsching, Germany), forming an array of 22 multidistant channels. Textile caps of different sizes were used according to the age and head size of the subjects. The probe arrangement was fixed in each of the caps using grommets, optode stabilizers, colored labels, and holders in order to assure comparable probe mapping over all subjects. Visual areas were identified according to the craniocerebral topography within the international 10-20 system and the placement of the optodes was done using fOLD v2.2 (Zimeo Morais et al., 2018) and NIRSite 2.0 (NIRx Medical Technologies LLC) software. Sources (8) and detectors (7) were symmetrically distributed to define 22 channels around the ROIs, each adjacent pair of sources and detectors defining one channel (min-max source-detector separation: 20–44mm for adults, 22–30mm for children; Fig. 3.1).

For data recording, the Aurora Software 1.4.1.1 (NIRx Medical Technologies LLC) was employed. The sampling rate was 10.2 Hz.

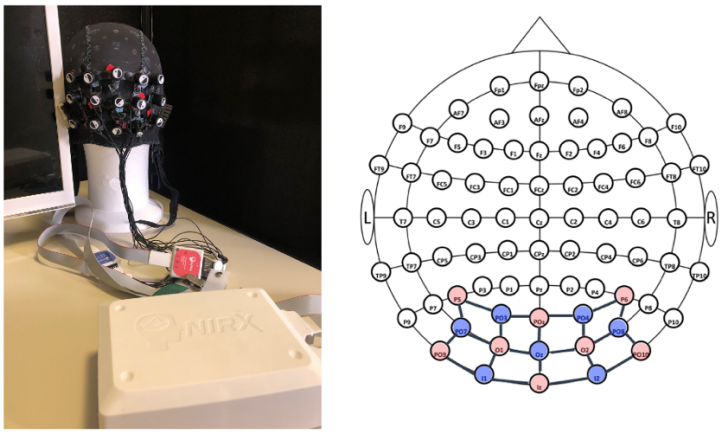

Figure 3.1 Occipital 8x7 montage. Optodes are placed according to the international 10-20 system; sources (red circles) and detectors (blue circles) were symmetrically distributed to define 22 channels around the visual areas of the cortex, each adjacent pair of sources and detectors defining one channel.

3.2 Task-related visual paradigm

Visual stimuli were generated using Python 3 and Psychopy3 (Peirce et al., 2019) and displayed with gamma correction on a monitor (Sharp LC-32LE352EWH, 60 Hz refresh rate, 45 cd/ m2 mean luminance, resolution of 800×600 pixels) placed 70 cm from the subject. Cortical hemodynamics in response to full-field, reversing, square wave, radial checkerboard, with abrupt phase inversion (spatial frequency: 0.33 cycles per degree, temporal frequency: 4 Hz; Fig. 3.2) was evaluated in the time domain by measuring the peak-to-baseline amplitude and latency. To increase the entertaining quality of the experimental paradigm, the checkerboard pattern was blended with an isoluminant commercial cartoon, thus serving as a reference baseline. To have an internal control with blank stimulation, we used an event-related design consisting of: (i) 20 cycles of 5 s stimulus 'on' (reversing checkerboard, 90% of contrast) followed by 10 s stimulus 'off' and (ii) 20 cycles of 5 s mock stimulus 'on' (reversing checkerboard, 0% of contrast) followed by 10 s stimulus 'off'. The two stimulating conditions were pseudo randomly interleaved for each subject during the recording. Blocks lasted 10 min and participants were permitted to take rest between recordings. Figure 3.2 shows a schematic representation of the experimental procedure. Visual events were synchronized with NIRSport over wireless LAN communication through the Python version of LabStreamingLayer (https://github.com/sccn/labstreaminglayer).

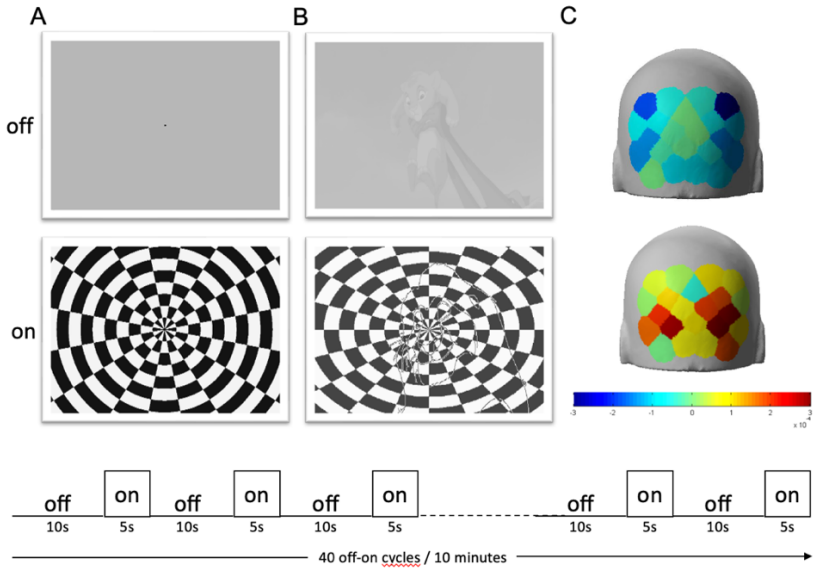

Figure 3.2 Visual stimulation and experimental paradigm. A-B Representative frame of baseline grey screen (upper row, stimulus 'off') and reversing checkerboard (lower row, stimulus 'on') for RS condition (**A**) and for Cartoon condition (**B**). **C** Representative HDR in the occipital cortex during the stimulus 'off' (upper row) and stimulus 'on' activation phase (lower row) according to the output of nirsLAB software. **D** Experimental protocol showing that the cycles of visual stimulation were structured in blocks of 40 trials (20 trials with the reversing checkerboard and 20 trials with the 'mock' stimulus) for a total duration of 10 min.

3.3 Signal processing and statistical analysis

Data preprocessing was completed using the Homer3 package (v1.29.8) in MATLAB (R2020a). A specific processing stream tailored on recent guidelines for the analysis of fNIRS data has been created (Di Lorenzo et al., 2019).

First, the raw intensity data were converted to optical density (OD) changes (hmR_Intensity2OD). Then, channels showing very high or low optical intensity were excluded from further analyses using the function hmR_PruneChannels (dRange: 5e-04-1e +00, SNRthresh: 2; SDrange: 0.0–45.0).

Motion artifacts were then removed by a multistep rejection protocol. After a step of motion artifact detection using the hmR_MotionArtifactByChannel function (tMotion: 1.0, tMask: 1.0; STDEVthresh 13.0; AMPthresh: 0.40), motion correction was performed with a combination of Spline interpolation (hmR_MotionCorrectSpline, p: 0.99) and Wavelet filtering (hmR_MotionCorrectWavelet, iqr: 0.80) functions. The remaining uncorrected motion artifacts were identified using the hmR_MotionArtifactByChannel function.

A band-pass filter (hmR_BandpassFilt: Bandpass_Filter_OpticalDensity, hpf:0.01, lpf:0.50) was applied to decrease slow drifts and high-frequency noise, and the OD data were converted to Hb concentration changes using the modified Beer–Lambert law (hmR_OD2Conc, ppf: 1.0 1.0 1.0).

Finally, trials of each subject were block-averaged for every stimulating condition and channel (hmR_BlockAvg: Block_Average_on_Concentration_Data, trange: −2.0 20.0). The resulting txt file was imported in Python as a Pandas DataFrame.

For each subject, only the channel with the highest response amplitude was analyzed. The peak response was identified as the maximal value for THb and OHb and the minimum value for DHb. A grand average was taken of the 20 trials of data per stimulating condition and differences between visual stimulation ‘on’ (reversing checkerboard) and ‘off’ (blank) were compared. All data were normalized with respect to the blank-evoked response using a subtraction method. Statistical analysis was carried out using pingouin Python library (Vallat, 2018) and the following functions: pingouin.ttest (paired and two-sided t-test), pingouin.rm_anova (one-way repeated measures ANOVA), pingouin.pairwise_ttests (post hoc analysis), pingouin.pairwise_corr (Spearman correlation), pingouin.regplot (Linear regression). T-test, ANOVA, and post hoc analysis were used to assess differences in fNIRS peak responses following different stimulating conditions, whereas we tested the interaction between the amplitude of fNIRS measures and selected clinical metrics with Spearman correlation.

3.4 Best practices for fNIRS experimental procedure

In order to achieve uniformity and quality of performance, while reducing bias, we defined a laboratory standard operating procedure (SOP) for visual fNIRS recordings. Indeed, a set of step-by-step instructions aims to define timeline of procedure, from preparing setting to recording, outlining specific rules within operators has been defined.

First of all, clinical staff manage recruitment phase to evaluate inclusion and exclusion criteria of eligible subjects and explaining exhaustively the procedure (a simple tutorial video of fNIRS recording is available to be shared with families); a previous interaction will be important to predict for each subject potential issues that might be encountered during the session and to make more efficient setting preparation phase (i.e., measuring in advance head circumference in order to choose the proper cap size). The technical staff periodically checked (once a month) the proper functioning of hardware and software (basically simulating an experiment) according to a specific calendar held in the room. Any issues (and possibly relative solutions) have been described in a dedicated journal. The technical staff also charged the fNIRS device periodically (need-based). The best choice is to perform the recordings with the device in battery mode (no wire). During experimental session two operators are required to be present (one member of the clinical staff and one member of the technical staff). The technical staff mainly managed the stimulation/acquisition procedures, the clinical staff was in charge of interactions with the subject and the parent (welcoming, general supervision of subject behavior). In the laboratory, after welcoming and brief introduction of the technical staff, the subject was invited to sit in the recording chair by the clinical staff (properly placed proper position around 70cm from the stimulating screen, the position is indicated by a white tape on the floor). For fNIRS recordings in children, the parent sit in a chair next to the subject. The clinical staff placed the textile cap on the head of the subject and startd the calibration process in order to preliminary verify level quality of each channel (Max tolerance for evoked recordings: 4 red flags/22 channels). fNIRS signal were recorded during consecutive trials of visually-evoked stimuli lasting about 10 minutes (with a resting time of about 5 minutes between each other required by the subject), tailored on study population as detailed in the next paragraphs. Operators are required to observe the global behavior of the subject (head movement, major movements, anxiety, etc.) and observing the hemodynamic signal in order to eventually note potential problems.

3.5 Recruitment and ethics statement

The whole research project was approved by Comitato Etico Pediatrico Regione Toscana Meyer (authorization number 201/2019 and 119/2021). Each one of the studies were conducted in accordance with the local legislation and institutional requirements. All the procedures on human participants were conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all adult participants and from the parents of each child, authorizing the use of anonymized data for research purposes. Assent was also obtained from the children involved in the study before participation.

Adults and typically developed children of control group were recruited as volunteers. Patients that usually are referred to IRCCS Stella Maris Foundation (SMF) to perform scheduled clinical visits were contextually evaluated for inclusion in the fNIRS study by referral clinicians. Indeed, the institute SMF has a robust experience in the field of neurodevelopmental disorders, including ASD and CTD thus facilitating the recruitment phase of this project, but also providing a set of clinical outcomes for the correlational analysis with fNIRS features.

In order to disseminate the project and promote the recruitment tailored communication material has been created and shared also with families' associations. Notably, in order to optimize compliance during experimental protocol, a wide online survey about the habits of children with CTD was conducted. Moreover, in parallel preliminary results of the project have been shared in academic field in order to raise awareness of the research topic (i.e., through the attendance at national and international conferences).

4 Results

4.1 Standardizing the technique in normative populations

4.1.1 Feasibility and validity of fNIRS visual HDR in adults and children with typical development

A sample of 40 adult subjects (20 women, age: 31.05 ± 3.94 (SD) years) and 19 children (5 girls, age: 7.20 ± 3.01 (SD) years) was recruited. All participants reported normal or corrected-to-normal vision and had no diagnosed neuropsychiatric condition. Adults and children underwent different experimental plans detailed in Fig. 4.1. Recordings in adult participants consisted in two type of experiments.

Experiment 1 (exp1) aimed to understand whether a reliable hemodynamic signal could be recorded in response to the radial checkerboard merged with an animated cartoon. Thus, exp1 started with a 10 min recording using the reversing checkerboard as stimulus 'on' and the gray screen as stimulus 'off' (RS condition), and continued with the vision of two different blended animated cartoons, where the stimulus 'on' was a merge between the reversing checkerboard and the movie, whereas the stimulus 'off' was the gray-scale isoluminant cartoon (CF and CC conditions; Fig. 4.1 panel A). The main purpose of using a cartoon was to increase the entertaining value of visual stimulation. The checkerboard presentation was needed to ensure a standardized episodic stimulation allowing event-related transient analysis. We decided to merge the checkerboard with the movie in order to avoid possibly distracting interruptions of the storyline and to facilitate screen fixation in children. The merging procedure was achieved using Python3 OpenCV (Gollapudi, 2019). Each pixel of the animated cartoon with the same color of the corresponding pixel of the radial checkerboard was inverted, to obtain a fully visible image. The result was a RS with an overlayed cartoon frame. The first cartoon was randomly selected by the operator within a group of 4 ("The Lion King", "The Powerpuff Girls", "Peppa Pig" or "Kung Fu Panda"; cartoon fixed, CF), whereas the latter was a free choice of the subject (cartoon chosen, CC).

Experiment 2 (exp2), aiming to dissect the contribution of baseline contrast to visual responses, was performed in a subset of adult participants (n =15). Exp2 consisted of

3 consecutive recordings of a CF ("Peppa Pig"; "Hide-and-seek", "Fly the kite", "Polly parrot" episodes) with the modulation of the baseline contrast (20, 40, and 80%; Fig. 4.1, panel B). The presentation order of different contrast levels was randomly shuffled.

In children, a single experiment was conducted (Fig. 4.1 panel C, exp1). To confirm that the baseline movie and its contrast do not affect the emergence of visual responses to the radial checkerboard, hemodynamic signals in response to 2 different blended RS-animated cartoons freely decided by the subject was recorded: cartoon 1 was presented at both low (20%, L1) and high (80%, H1) contrast, while only low contrast was recorded for cartoon 2 (L2; Fig. 4.1 panel C). In this case, the presentation order was decided by the child, in order to maximize subject compliance.
During the experimental session, data were quickly analyzed and visualized using nirsLAB software (NIRx Medical Technologies LLC, v2019.4) in order to immediately monitor quality of recording or early detect and fix potential issues.

In order to investigate the potential correlation between fNIRS signal and broad behavioral dimensions in the general population, we asked to participants to fill out the Autism-Spectrum Questionnaire (AQ), respectively the adult and the child form (Baron–Cohen et al., 2001; Auyeung et al., 2008). The AQ questionnaire is a 50-items 4-point Likert scale self-administered report (or parent-report for child version), validated for the Italian version (Ruta et al., 2012), consisting of descriptive statements assessing personal preferences and typical behavior. The items are grouped in five subscales: Social Skills, Communication, Attention to Details, Imagination, and Attention Switching. For adult version, total scores range between 0 and 50 (0–10 for each subscale), with 32 being the clinical threshold for autism risk (Baron-Cohen et al., 2001). For child version, total scores range between 0 and 150 (0–30 for each subscale), with 76 being the clinical threshold for autism risk (Auyeung et al., 2008).

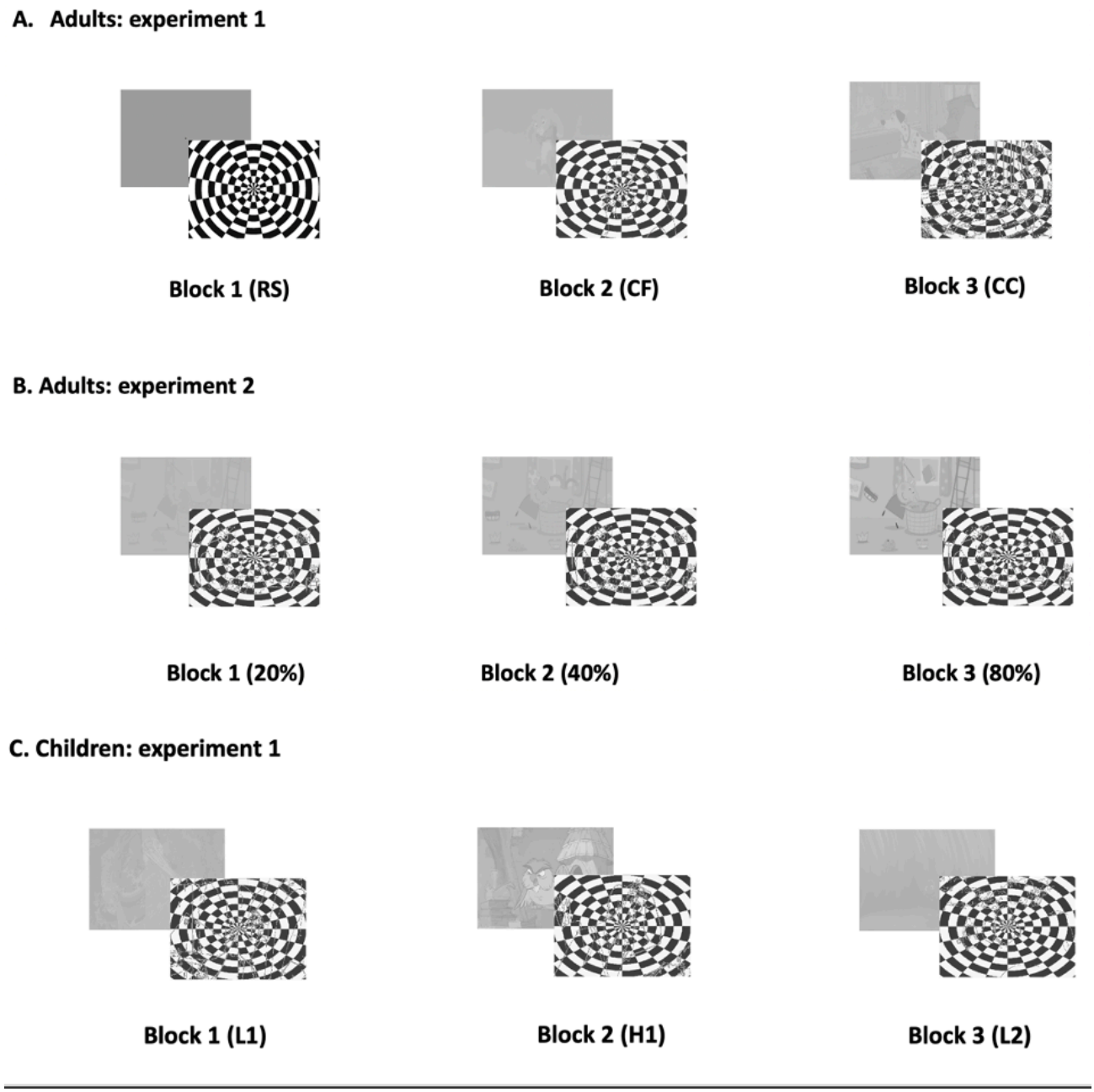

Figure 4.1 Experimental plan. For each block (i.e., stimulating conditions), representative frames of baseline screen (stimulus 'off', up) and reversing checkerboard (stimulus 'on', low) were shown. **A** Experiment 1 for adult participants consisted in three 10-minute blocks, starting with the vision of the classic radial checkerboard-on-grey stimulus (RS condition) and continuing with two different RS blended animated cartoons. The first cartoon was decided by the operator within a group of 4 (CF condition), whereas the latter was a free choice of the subject (CC condition). **B** Experiment 2 was carried out in a subset of adult participants (n = 15), watching the same «Peppa Pig» episodes with the modulation of the baseline contrast (20%, 40&, 80%). **C** Children were recorded during the vision of 2 different blended animated cartoons freely selected by the subject: cartoon 1 was shown at both low (20%, L1) and high (80%, H1) contrast, while only low contrast was used for cartoon 2 (L2).
An example of experimental visual stimulation protocol could be find online at the following link: https://drive.google.com/file/d/17PxviLZWhNHiuBtGPh41Icmlwp7xaYaw/view?usp=share_link

4.1.1.1 *An animated cartoon-based stimulus is able to evoke visual responses in the adult cortex*

The cortical HDR function elicited by a reversing checkerboard pattern was measured in the adult population (see Tab. 1 for demographics in the Appendix). In agreement with the previous literature (Chen et al., 2015), a significant activation of the occipital cortex in response to different conditions of visual stimulation was obtained. Grand averages across adult participants of THb, OHb and DHb concentration changes are plotted in Fig. 4.2. Using a classic mean luminance gray screen as baseline, statistical analysis revealed a significant main effect of the checkerboard stimulus (S) with respect to the blank presentation for all HDR metrics (Radial Stimulus condition, RS, Fig 4.2, panel A). As previously discussed, to increase the entertaining quality of the experimental paradigm, an innovative visual stimulation protocol blending the checkerboard pattern with an isoluminant commercial cartoon, thus serving as a reference baseline, was devised. A significant increase of THb and OHb, with a parallel reduction of DHb concentration in response to S appearance was found, reflecting the functional activation of visual areas in this condition as well. The cortical response was independent from the cartoon employed as baseline: a comparable HDR, indeed, was clearly elicited both when the baseline movie was fixed a priori by the experimenter (Cartoon Fixed condition, CF; Fig. 4.2, panel B) and when the cartoon was freely selected by the tested subject (Cartoon Chosen condition, CC; Fig. 4.2, panel C). The amplitude of cortical activation was only slightly smaller in response to CF and CC, indicating that the quality of visual input does not quantitatively impact HDR, with the range of OHb and DHb fluctuations being significantly lower with respect to that evoked by RS (Fig. 4.2, panel D). Within the CF condition, it can be assumed that the baseline cartoon does not affect the degree of visual activation: indeed, a comparable modification of THb, OHb, and DHb concentrations was recorded using the four cartoons chosen a priori. Furthermore, no differences of visually evoked responses were detected modulating the contrast level of the baseline cartoon: THb, OHb, and DHb fluctuations, indeed, were comparable when a fixed baseline cartoon was presented at 20, 40, or 80% of contrast (Fig. 4.2, panel E). Finally, the response latency was homogenous in RS, CF, and CC conditions (Fig. 4.3, panel A).

Altogether, these results demonstrate the validity of this innovative stimulation procedure to evoke a significant and reliable response in the occipital cortex preserving inter-subject variability.

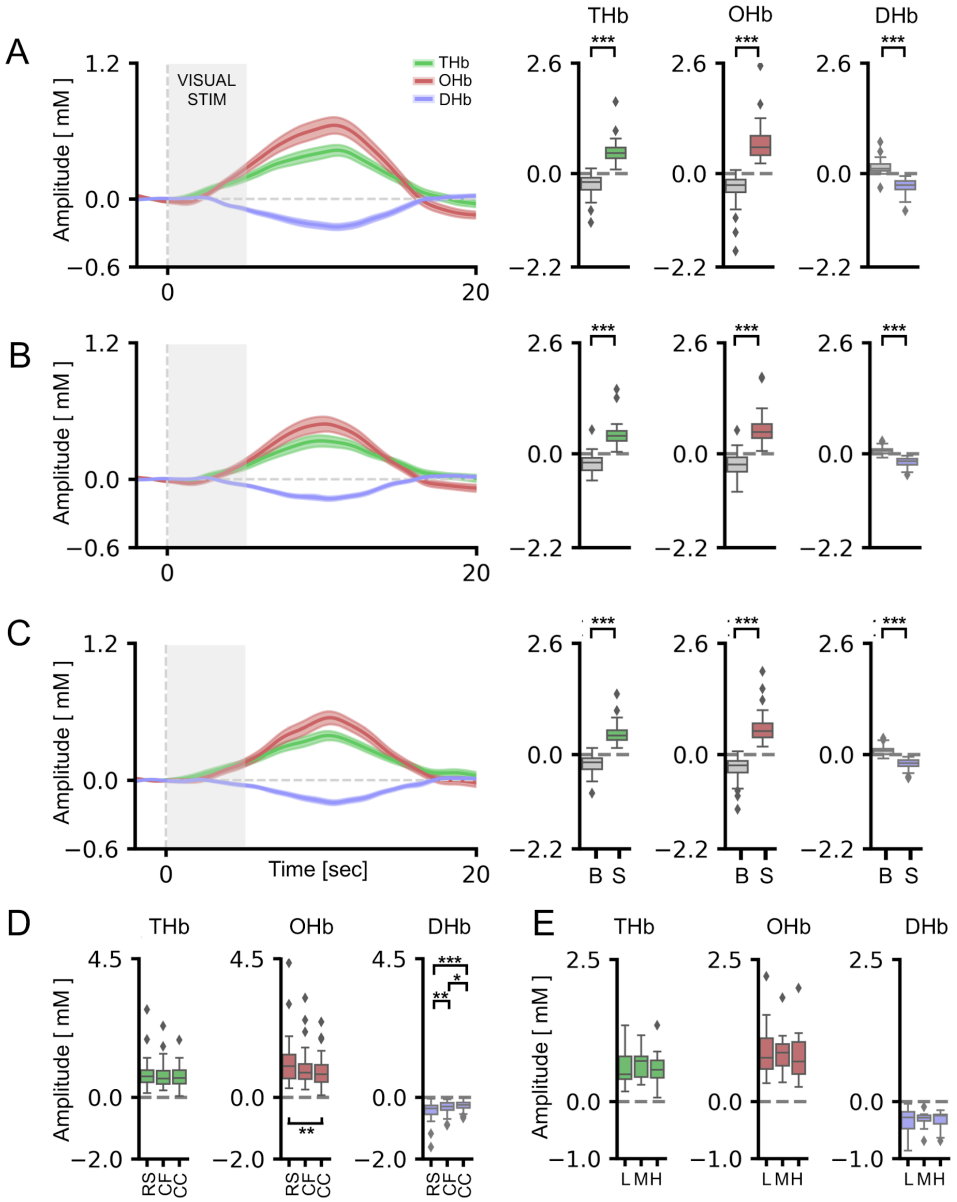

Figure 4.2 HDR was reliably detected in adults using both RS and blended RS-animated cartoons. For all panels, values in the y-axis are multiplied for 10^4. **A** On the left, the average time course for THb (green line), OHb (red line), and DHb (blue line) in response to the Radial Simulus (RS) are shown. The three plots on the right depict the average peak response to the stimulus (S) vs. the blank (B) across all the adult subjects. The stimulus-driven signal was significantly different from the blank for all the conditions (t-test, $p < 0.001$ for all comparisons). **B** Same plots as above for the Cartoon Fixed (CF) condition. On the left, the average time course of the evoked HDR is depicted. On the right, the graphs showed that the HDR amplitude was significantly higher in response to S with respect to B for THb, OHb, and DHb (t-test, $p < 0.001$ for all comparisons). **C** Cartoon Chosen (CC) condition. Also in this case the S elicited significantly higher responses for THb, OHb, and DHb with respect to the B (t-test, $p < 0.001$ for all comparisons). **D** Comparison among different visual stimulations shows no differences in evoked amplitudes for THb, whereas a significant difference was detected between RS and CC for OHb (One-way RM ANOVA, $p < 0.01$, post hoc BHFDR, RS vs. CC $p < 0.01$) and a more complex pattern of differences emerged for DHb (One-way RM ANOVA, $p < 0.001$, post hoc BH-FDR, RS vs. CF $p < 0.01$, RS vs. CC $p < 0.001$, CF vs CC $p < 0.05$). **E** No differences of evoked responses were detected with different contrast levels of the baseline movie (L: low, M: medium, H: high).. Data are shown as average ± s.e.m. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

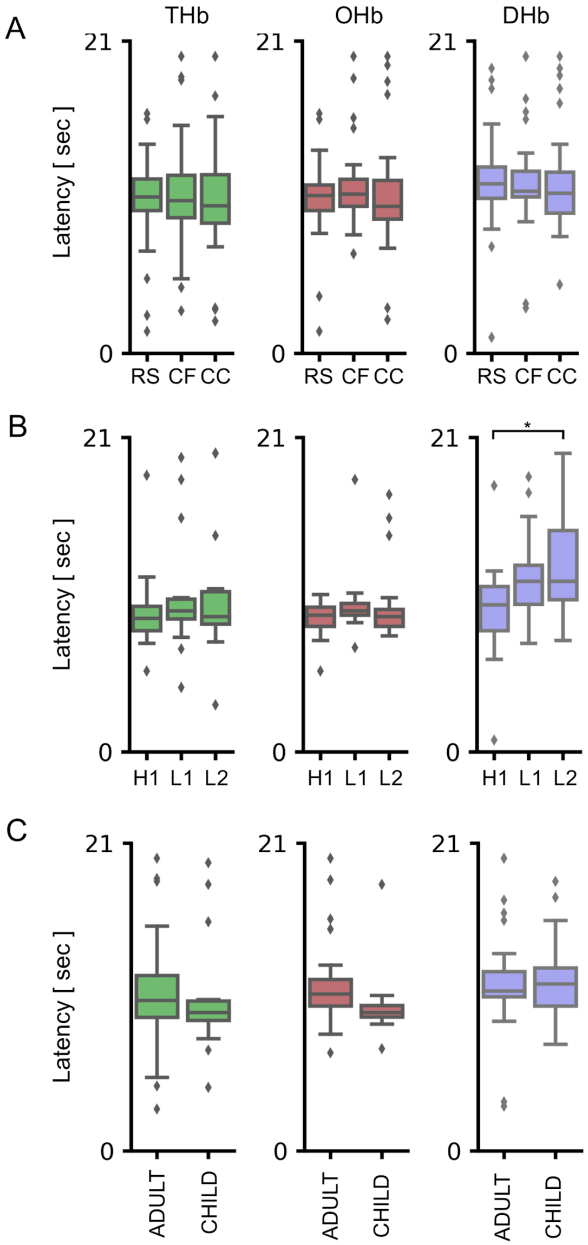

Figure 4.3 Latency of HDR in adults and children. A The analysis of latency to peak in adults revealed no differences among RS, CF and CC. **B** In children, no differences of HDR latencies were found between H1 and L1, whereas a significantly higher latency of DHb was detected between H1 and L2 (One-way RM ANOVA, $p < 0.05$, post hoc BH-FDR H1 vs. L2 $p < 0.05$). **C** No significant differences were identified for latencies between adults and children. * $p < 0.05$.

4.1.1.2 The cartoon-based paradigm was reliable in eliciting cortical responses in children

We measured cortical responses in typically developing children (see Tab. 2 for demographics in the Appendix) viewing the radial checkerboard blended with the animated cartoon consistently with experimental pipeline represented in Figure 4.1 (panel C). We compared three different conditions: each subject, indeed, was asked to select two cartoons of their preference for the baseline, and the first choice was employed for the low-contrast (cartoon 1 low contrast, 20%, L1) and the high-contrast (cartoon 1 high contrast, 80%, H1) stimulation, while the second cartoon was presented only at low-contrast (cartoon 2 low contrast, L2).

Our data showed a significant activation of the visual cortex, with a prominent change of THb, OHb, and DHb concentration in response to the S with respect to the blank for all conditions tested (Fig. 4.4, panel A–C). The amplitude of elicited cortical responses was comparable following L1, H1, and L2 (Fig. 4.4, panel D), proving that the HDR is independent from the cartoon narrative selected for the baseline and the contrast level of baseline presentation in children as well. Small and not statistically significant differences were observed for response latency among L1, H1, and L2 conditions (Fig. 4.3, panel B).

In agreement with previous literature (Ward et al., 2015), a maturational trend of cortical responsivity was recognized, with children showing significantly higher HDR amplitude with respect to adult subjects (Fig. 4.4, panel E).

These findings establish a novel method for measuring visually evoked cortical activity with fNIRS that ensures an elevated compliance of young subjects and high-quality reliability of measurements, suggesting a valuable tool for studying visual cortical processing in typically developing children, but also in clinically relevant populations.

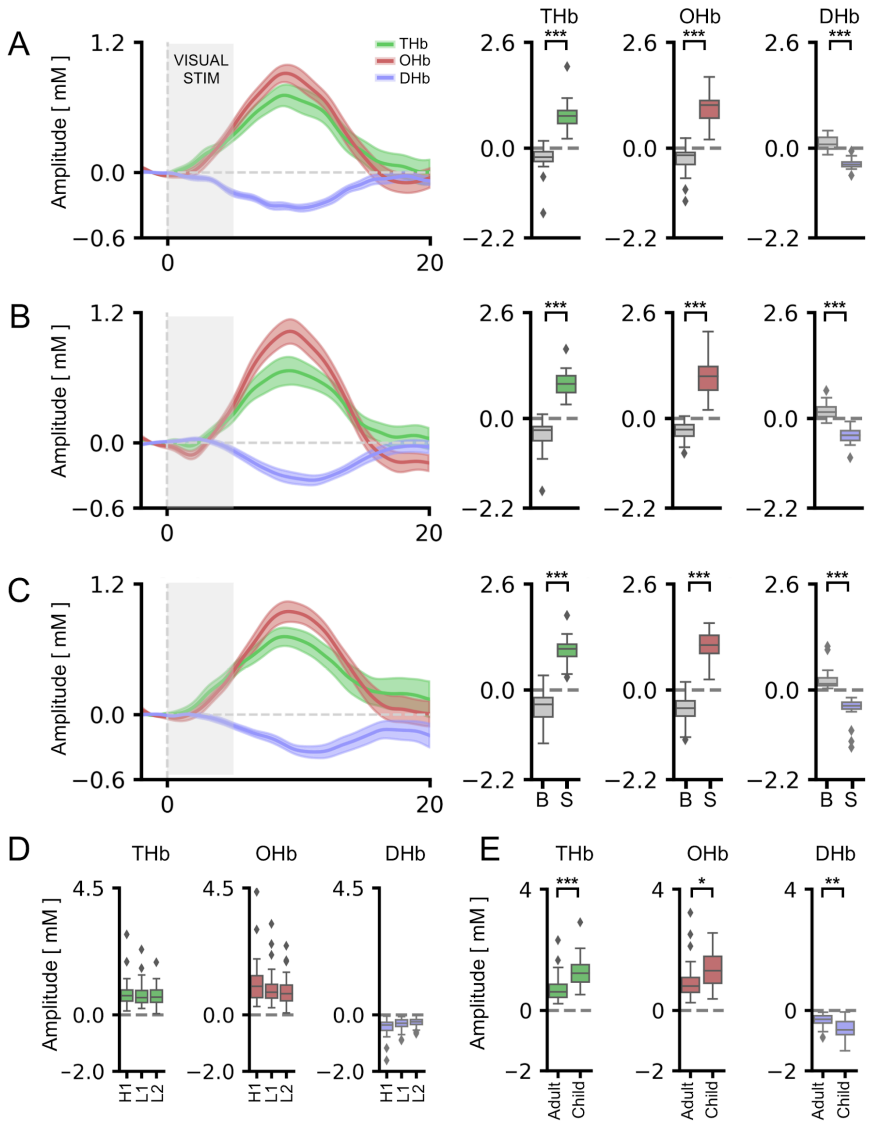

Figure 4.4 HDR signal was reliably detected in children using blended RS-animated cartoons with low and high contrast. For all panels, values in the y-axis are multiplied for 10^4. **A** On the left, the average time course for THb (green line), OHb (red line), and DHb (blue line) in response to high-contrast (80%) blended RS-animated cartoons is shown (CH1). On the right, the graphs represent the average amplitude of the evoked HDR following the stimulus (S) and the blank (B). A significantly different response to S with respect to the B was detectable for all metrics (ttest, $p < 0.001$ for all comparisons). **B** The average time course of the HDR to low-contrast (20%) blended RS-animated cartoons is shown (CL1). Here, the baseline cartoon is the same as the experiment described in panel (A), but a different part of the movie was used. THb, OHb, and DHb showed a significantly higher deflection to the S with respect to the B in this condition as well (t-test, $p < 0.001$ for all comparisons). **C** On the left, the average time course of HDR following the second low-contrast blended RS-animated cartoon selected by the subject (CL2). On the right, the analysis of peak amplitudes revealed significantly higher responses during S compared to B for THb, OHb, and DHb (t-test, $p < 0.001$ for all comparisons). **D** Comparison among different contrast levels of the baseline cartoon revealed no differences in the amplitude of HDR. **E** Response amplitudes for low-contrast blended RS-animated cartoons in adults and children. More specifically, we compared the response to CF condition of adults with CL1 condition for children. The average amplitude of HDR was significantly higher in children (t-test, $p < 0.001$ for THb, $p < 0.05$ for OHb, $p < 0.01$ for DHb). Data are shown as average ± s.e.m. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.1.1.3 The amplitude of fNIRS hemodynamic response in the visual cortex unmasks autistic traits in typically developing children

Moreover, a significant negative correlation of HDR amplitude with AQ score in children was found. Despite no effects detectable in adults (Fig. 4.5 A-C), the amplitude of visual responses was highly correlated to AQ scores in children (Fig. 4.5 D-E). Consistent with a recent work (Xu et al., 2020), the correlation was specific for THb, with higher AQ score being associated with a lower amplitude of THb visually-evoked signals. Interestingly, HDR amplitude was especially linked to social and communication autistic traits (Fig. 4.5 panel A-D): indeed, assessing separately the five AQ subscales (Auyeung et al., 2008) a significant correlation of THb and OHb with the Social Skills subscale was found (AQ_S, Fig. 4.6 panel A-B), while only THb modulation was related to the Communication subscale (AQ_C, Fig. 4.6 panel C-D) and no significant interaction was observed testing the other three AQ subscales (AQ_A, AQ_D, and AQ_I; Fig. A1 in the Appendix). Given the reliability across different visual tasks (L1 and H1), the strongest interaction was between THb and AQ_S (Fig. 4.6 panel A-B, THb metric shown in green).

These data were published in February 2022 on Translational Psychiatry 12(1): 53 (Mazziotti et al., 2022).

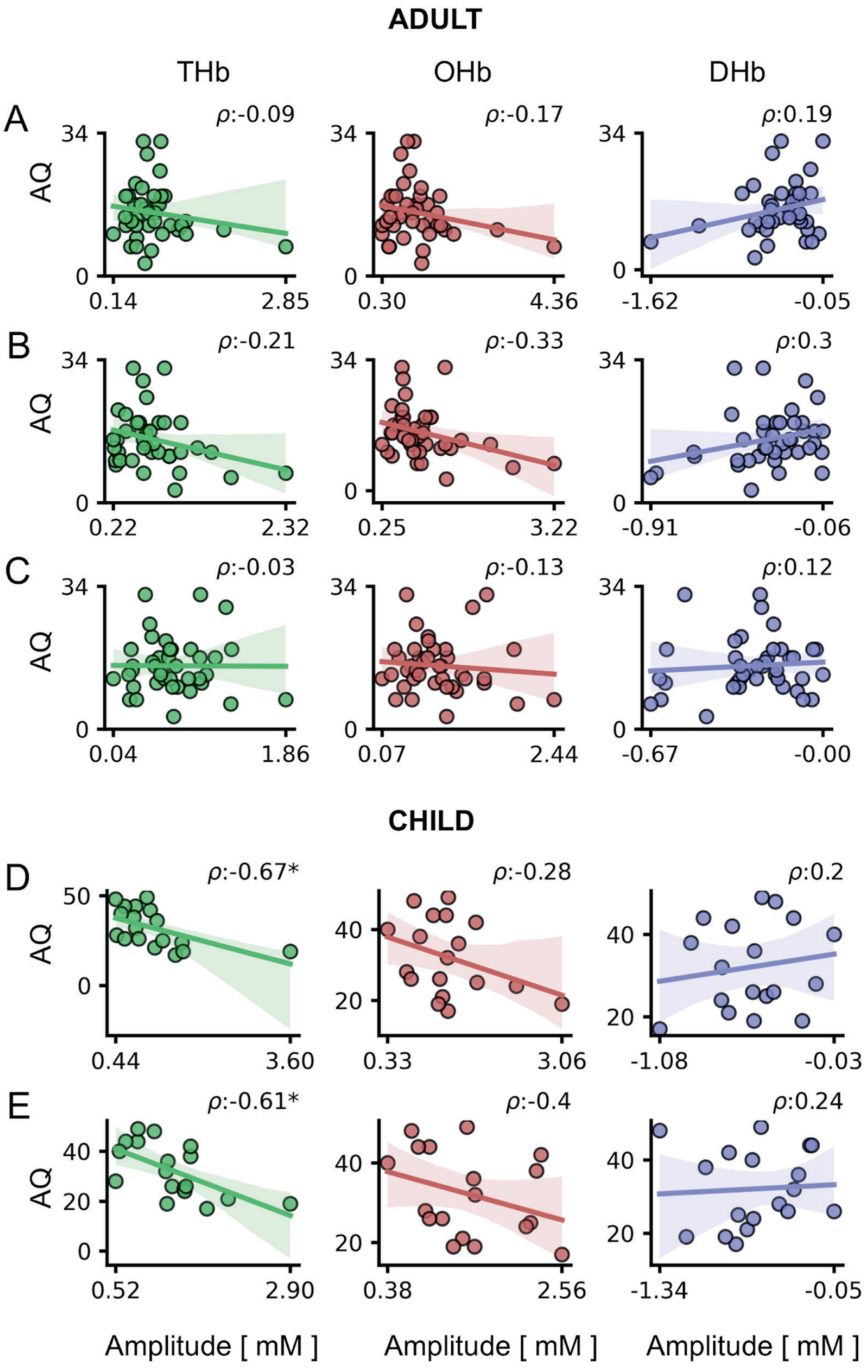

Figure 4.5 Correlation between HDR and AQ scores. For all panels, values in the x-axis are multiplied for 10^4. The ρ (rho) index in each plot indicates the Spearman correlation value. Correlation between HDR and AQ scores in adults, for amplitudes obtained using RS (**A**), CF (**B**), and CC (**C**). No significant correlations were detected for adult participants. Correlation between HDR and AQ scores in children, for amplitudes obtained using high (**D**), and low (**E**) contrast baseline cartoons. A significant correlation was found between THb and AQ scores for both high and low-contrast blended stimuli ($p < 0.05$ for both cases). Circles are individual values; lines represent the linear regression model fit and shaded regions are the 95% CI.

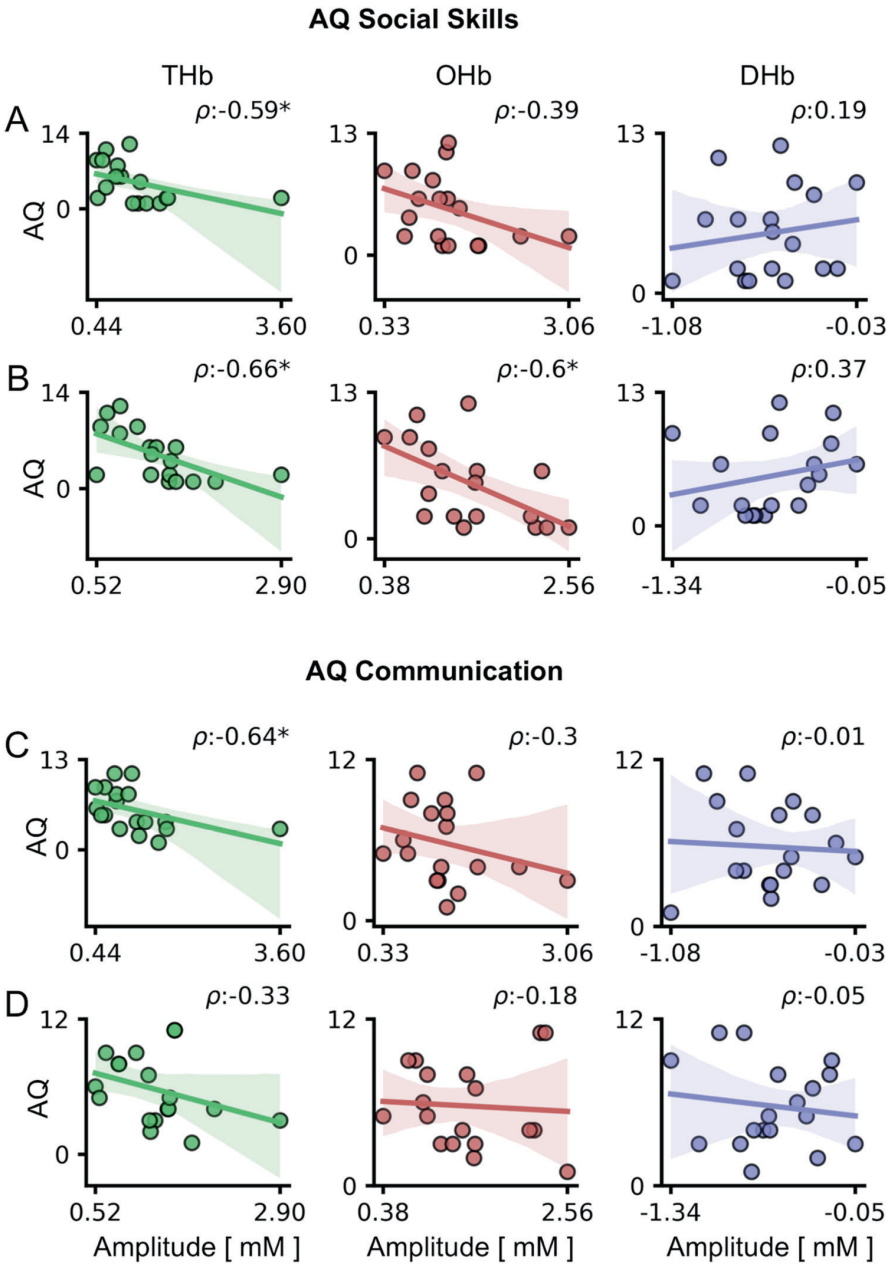

Figure 4.6 between HDR and AQ subscales score (panel C-F) in children. For all panels, values in the x-axis are multiplied for 10^4. The ρ (rho) index in each plot indicates the Spearman correlation value. Circles are individual values; lines represent the linear regression model fit and shaded regions are the 95% CI. Correlations between HDR and AQ Social Skills (AQ_S) subscale are shown in panel **A-B**. A significant correlation between THb and AQ_S was detected using both high- (A) and low-contrast blended stimuli (B; $p < 0.05$ for both cases). In addition, OHb recorded in response to the low-contrast blended RS-cartoon was significantly correlated with AQ_S (C; $p < 0.05$). Correlations between HDR and AQ Communication (AQ_C) subscale are shown in panel **C-D**. THb amplitude in response to the high-contrast blended RS-cartoon was significantly correlated with AQ_C ($p < 0.05$).

4.1.2 Assessing test-retest reliability of fNIRS vHDR paradigm

Reliability and reproducibility of a specific experimental paradigm are two critical features for establishing the validity of a biomarker.

Previous reports have already suggested that fNIRS could be a cortical be a reliable measure of visual (Plichta et al., 2006), motor (Strangman et al., 2006) and executive (Schecklmann et al., 2008) functions. These studied have been conducted mainly in adult populations but more sporadically test – retest validity of fNIRS measurement was assessed also in younger cohorts (Blasi et al., 2014).

Here, a systematic evaluation of rest-retest reliability of fNIRS vHDR metric has been performed in order to confirm its potential validity as a biomarker in NDDs cohort.

Fifty healthy volunteers (25 women and 25 men) performed two recording sessions for the present study. Three intervals to assess test-retest reliability of our measurement were established: a short-term repetition of fNIRS recording (intrasession: timepoint 0-1) and two longer retest intervals (intersession: timepoint 0-2 at 2 months and timepoint 0-3 at around 2 years). Thus, all participants (50 subjects) were recorded twice in a single session to evaluate short-term test-retest reliability of fNIRS vHDR metric. Moreover, 23 adult subjects of our first cohort (12 women; mean age at enrollment date: 31.6 $\pm$ 4.1 (SD) years) were recruited for long-term test-retest reliability and 27 new healthy adult volunteers for mid-term test-retest reliability (13 women; mean age: 30.4 $\pm$ 4.3 (SD) years). Even if a negligible circadian variability of fNIRS response in primary visual cortices has been previously reported (Schroeter et al., 2006), suggesting the robustness of this stimulus-driven metric, all the recording were conducted almost at the same hour in the afternoon.

All subjects had normal or corrected to normal vision. No subject had a known history of any neurologic or psychiatric disorder. All subjects were informed about the nature of the experiment as well as the operating mode of the NIRS instrument, before giving their written informed consent. Recordings were conducted as described in Material and Methods section. The visual stimulation was realized in the event-related paradigm previously described by presenting a simple checkerboard blended with an isoluminant commercial cartoon presented at 20% contrast level. Consistently with previous experience, number of trials was set to n = 20. Data analysis was conducted with pipeline described in Material and Methods.

The groupwise intrasession reliability of fNIRS measurement resulted to be very good for all the three metrics: indeed, the correlation between the amplitude recorded at

timepoint 0 and the signal magnitude at timepoint 1 was highly significant both for average values (Fig. 4.7) and best channel values (Fig. 4.8) for all the metrics.
In addition we examined the data calculating the mean difference for each pair of recordings. Despite a slight tendency to increased OHb amplitude, our analysis highlighed that this difference is centered on the zero level for intrasession interval (Fig. 4.9). Similar results were found analyzing both average values (Fig. 4.9, panel A-C) and best channel values (Fig. 4.9, panel D-F) of vHDR metrics (THb, OHb, DHb).
More importantly, the same consistency of results was found for intersession reliability of vHDR signal. Even if the analysis of the timepoint 0-2 is still not complete and data shown are only preliminary, the correlation of signal amplitudes across the timepoint 0-3 (Figures 4.7 and 4.8) was significant for the metrics tested, mostly for best channel analysis. Furthermore, the longitudinal analysis of paired differences did not highlight any significant discrepancy of signal distribution, neither for the 0-2 timepoint nor for the 0-3 interval for all the metrics tested (Figures 4.10 and 4.11).

Altogether these results demonstrated the high intra-subject accordance between two different vHDR measurements supporting the strong reliability of this approach.

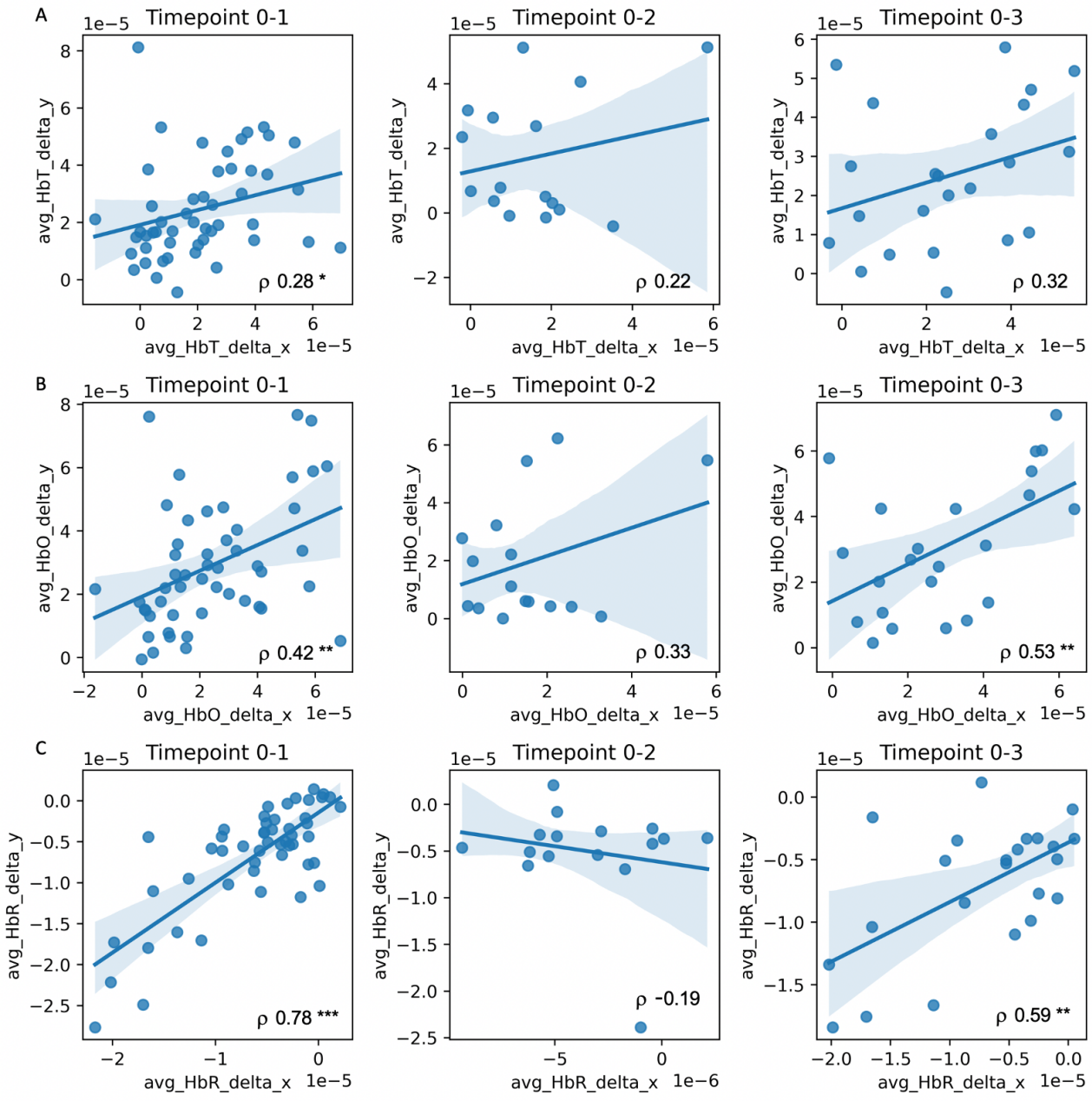

Figure 4.7 Scatter plot of the average values at group-level for THb (panel A), OHb (panel B) and DHb (panel C) at three subsequent timepoints. For all panels, values in the x-axis and in the y-axis are multiplied for 10^5. The ρ (rho) index in each plot indicates the Spearman correlation value. Circles are the average values of vHDR for each subject. Lines represent the linear regression model fit and shaded regions are the 95% CI. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Timepoint 0-1 refers to short, intrasession timepoint. Timepoint 0-2 refers to middle (2 months interval) intersession timepoint. Timepoint 0-3 refers to long (2 years interval) intersession timepoint. Analysis of timepoint 0-2 is preliminary, including only 16 upon 27 subjects recruited.

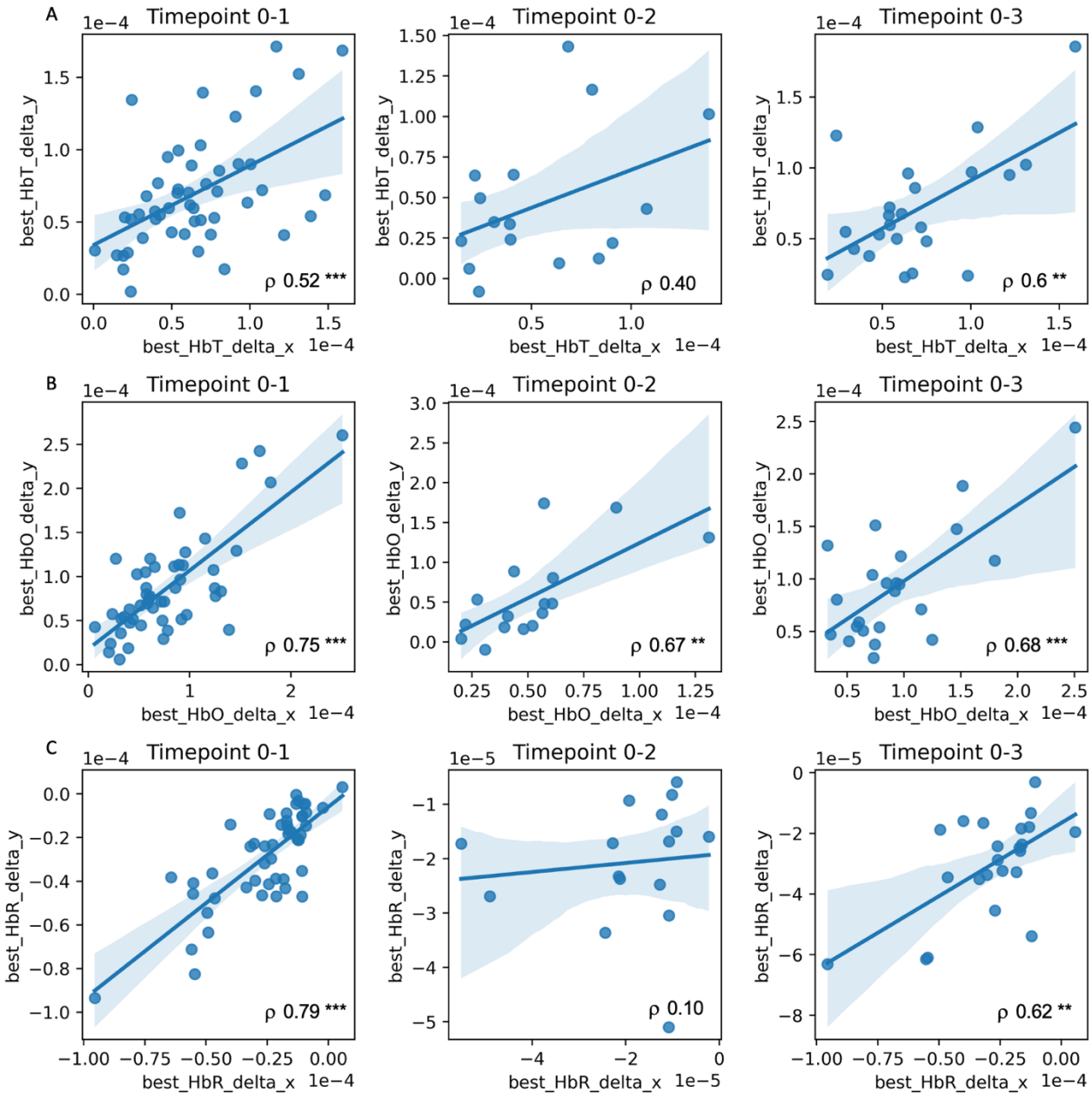

Figure 4.8 Scatter plot of the best channel values for THb (panel A), OHb (panel B) and DHb (panel C) at three subsequent timepoints. For all panels, values in the x-axis and in the y-axis are multiplied for 10^4. The ρ (rho) index in each plot indicates the Spearman correlation value. Circles are the value of vHDR in the higher amplitude channel (best) for each subject. Lines represent the linear regression model fit and shaded regions are the 95% CI. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Timepoint 0-1 refers to short, intrasession timepoint. Timepoint 0-2 refers to middle (2 months interval) intersession timepoint. Timepoint 0-3 refers to long (2 years interval) intersession timepoint. Analysis of timepoint 0-2 is preliminary, including only 16 upon 27 subjects recruited.

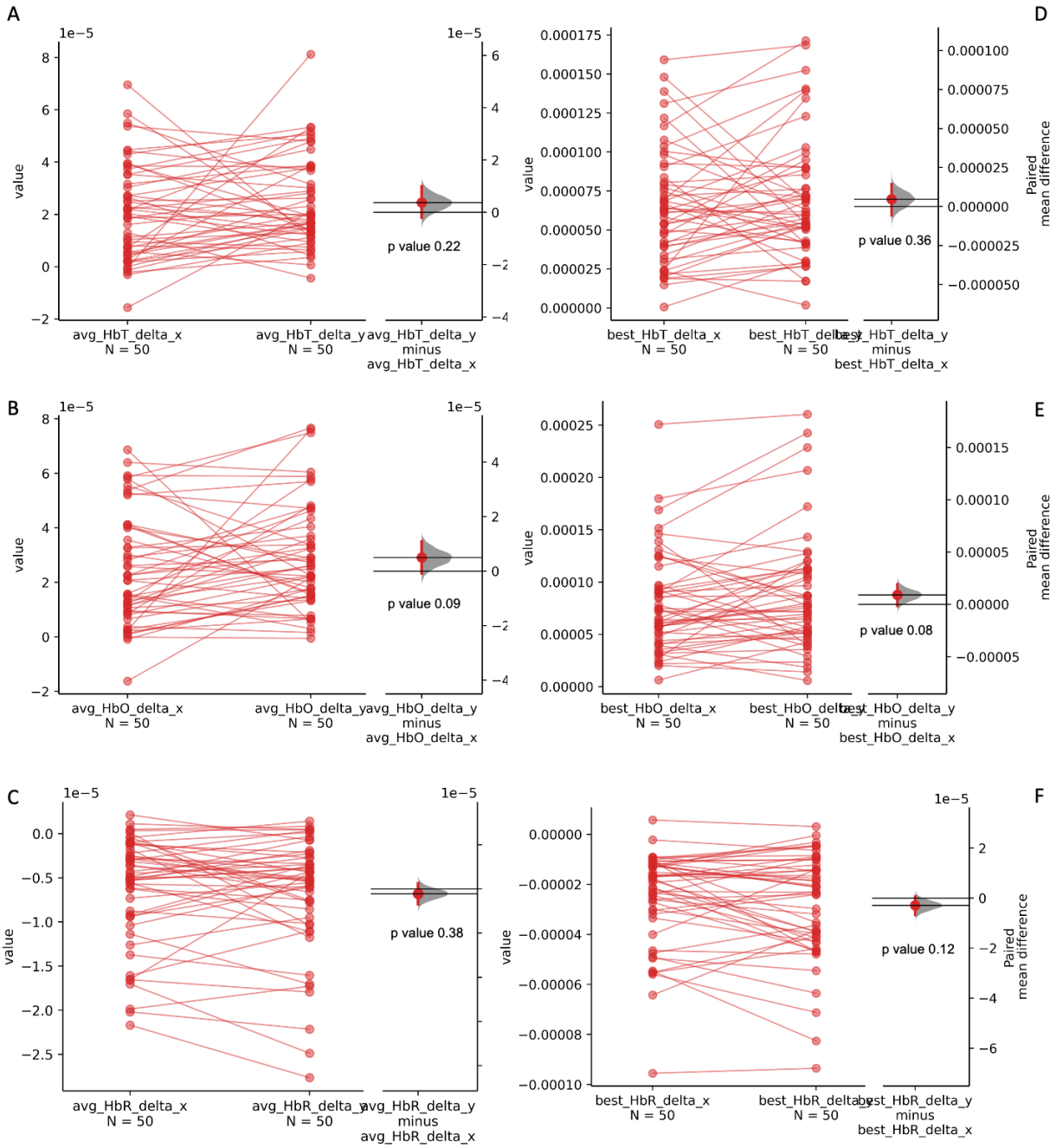

Figure 4.9 Intrasession test-retest reliability of vHDR metrics measured by paired men difference: average (panel A-C) and best channel (panel D-F) value. For all panels, values in the y-axis are multiplied for 10^5. Red dots represent average value (panel A-C) or best channel value (panel D-F) of vHDR magnitude values for each subject in the two intrasession recordings. The p-value of the two sided permutation t-test is reported for each metric on the right of the panel (CI 95%).

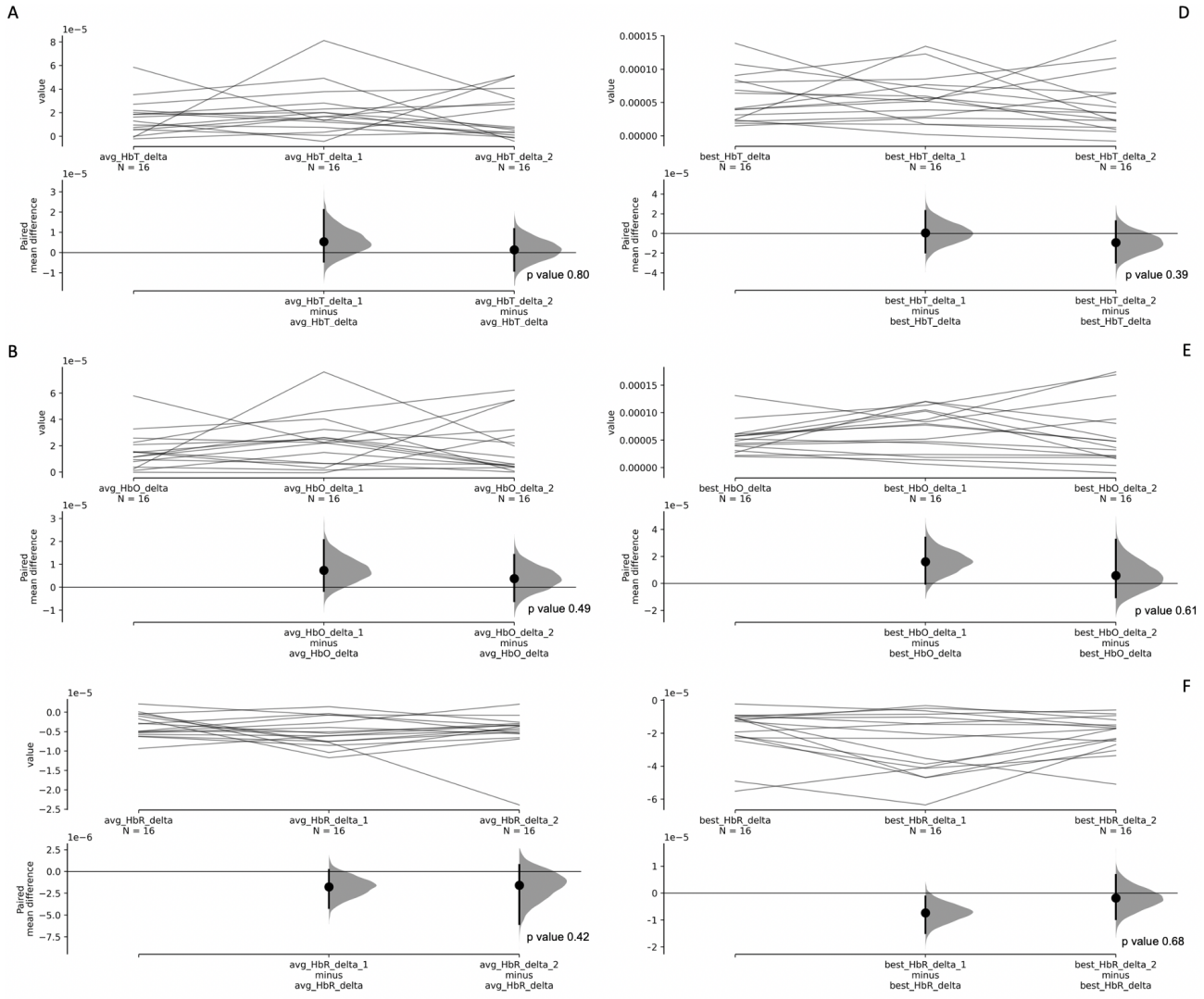

Figure 4.10 Intersession test-retest reliability of vHDR measured by paired men difference at midterm timepoint (time interval of 2 months): average (panel A-C) and best channel value (panel D-F) of all metrics. For all panels, values in the y-axis are multiplied for 10^5. Longitudinal representation of paired mean difference between average value (panel A-C) or best channel value (panel D-F) of vHDR magnitude values for each subject within intersession interval 0-2. The p-value of the two sided permutation t-test is reported for each metric on the right of the panel (CI 95%). Analysis of timepoint 0-2 is preliminary, including only 16 upon 27 subjects recruited.

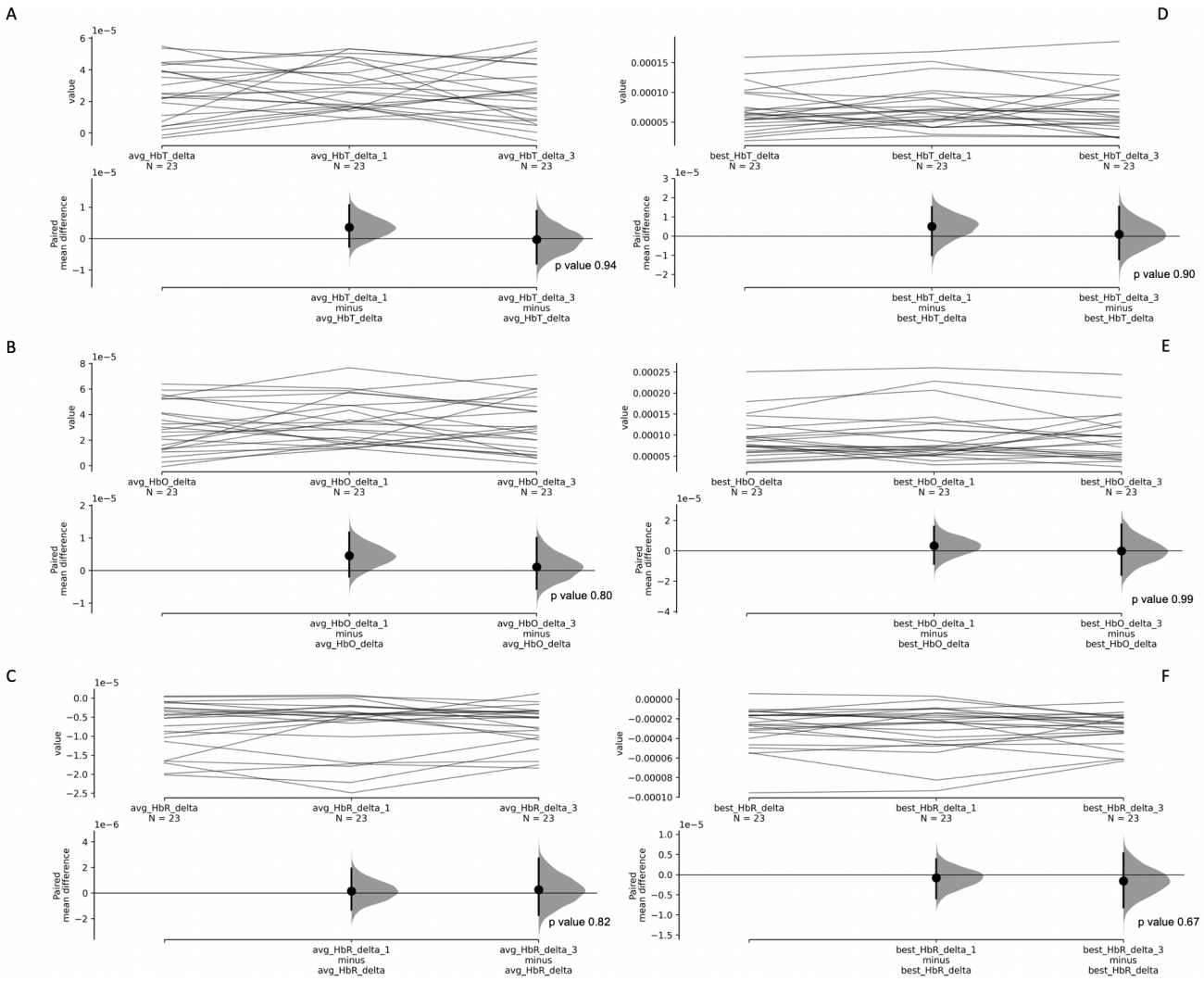

Figure 4.11 Intersession test-retest reliability of vHDR measured by paired men difference at long timepoint (time interval of 2 years): average (panel A-C) and best channel value (panel D-F) of all metrics. For all panels, values in the y-axis are multiplied for 10^5. Longitudinal representation of paired mean difference between average value (panel A-C) or best channel value (panel D-F) of vHDR magnitude values for each subject within intersession interval 0-3. The p-value of the two sided permutation t-test is reported for each metric on the right of the panel (CI 95%).

4.2 Testing feasibility and validity of fNIRS vHDR as a brain biomarker in NDDs

4.2.1 Exploring Autism spectrum Disorder (ASD) in preschooler females

Starting from previously described experience on TD children, showing the correlation of the metric with broad autistic behavioral traits, it has been hypothesized that visually-evoked hemodynamic responses (vHDR) might represent a quantitative and unbiased biomarker to assist the clinical assessment of ASD. In particular, female phenotype was studied, since it is more challenging in clinical practice due to the well-known camouflaging issue (Young et al., 2018).

Thus, a pilot study on a cohort of high-functioning preschooler females with idiopathic ASD (fASD) was performed with these specific aims:

- to test the feasibility of fNIRS vHDR procedure previously described in this clinical population;
- to investigate the validity of vHDR as an effective tool to discriminate between TD preschool girls and sex/age matched peers with ASD;
- to explore whether vHDR metrics correlate with clinical variables.

A cross-sectional observational monocentric study was conducted at the IRCCS Stella Maris Foundation Hospital (Pisa, Italy). A total of 12 females with idiopathic ASD (fASD: median age 4.5 years; SD: 1.17) and 13 sex/age-matched control typically developed peers (TD: median age 4.86; SD: 1.17) were recruited. Established inclusion criteria were: (1) a diagnosis of ASD according to the DSM-5 criteria (APA, 2013); (2) age-range: between 3 and 6 years; (3) female gender; (4) a negative history for premature birth or neurologic complications possibly related to delivery; and (5) non-verbal IQ $\geq$ 70. An ASD diagnosis was performed by a multidisciplinary team according to good clinical practice. The demographic characteristics and clinical features of experimental cohorts are listed in Tables 3 and 4 in the Appendix section. Clinical measures such as the Autism Diagnostic Observation Schedule (ADOS) (Lord et al., 2016), the children's version of the Autism Questionnaire (AQ) (Ruta et al., 2012), and a cognitive and adaptive profile were systematically collected for each subject using internationally validated scales/interviews, such as the Wechsler Preschool and Primary Scale of Intelligence (Freeman, 2013) and the Vineland Adaptive Behavior Scales–II–VABS (Sparrow et al., 2012).

4.2.1.1 A cartoon-based stimulus is able to evoke reliable responses in the visual cortex of fASD

Cortical vHDR elicited by a reversing checkerboard pattern merged with an isoluminant commercial cartoon (contrast level 0.8) was recorded by fNIRS device. In agreement with previous results, we obtained a significant activation of the occipital cortex in response to the stimulus in TD children. Grand averages across control participants for Total Hb (THb), Oxygenated Hb (OHb) and Deoxygenated Hb (DHb) concentration changes are plotted in Figures 4.12 and 4.13, showing the average amplitude of signals recorded in the different channels of the montage (Fig. 4.12, panel A–C) and the amplitude of the channel with the highest response within subjects (Fig. 4.13, panel A–C), respectively. A statistical analysis revealed a significant main effect of the checkerboard stimulus (S) with respect to the presentation of the mock stimulus (MS) for all HDR metrics. Importantly, a significant vHDR, with a prominent change of THb, OHb, and DHb concentration in response to the S with respect to the blank for all conditions tested, was also present in the fASD cohort (Fig. 4.12, panel D–F and Fig. 4.13, panel D–F).

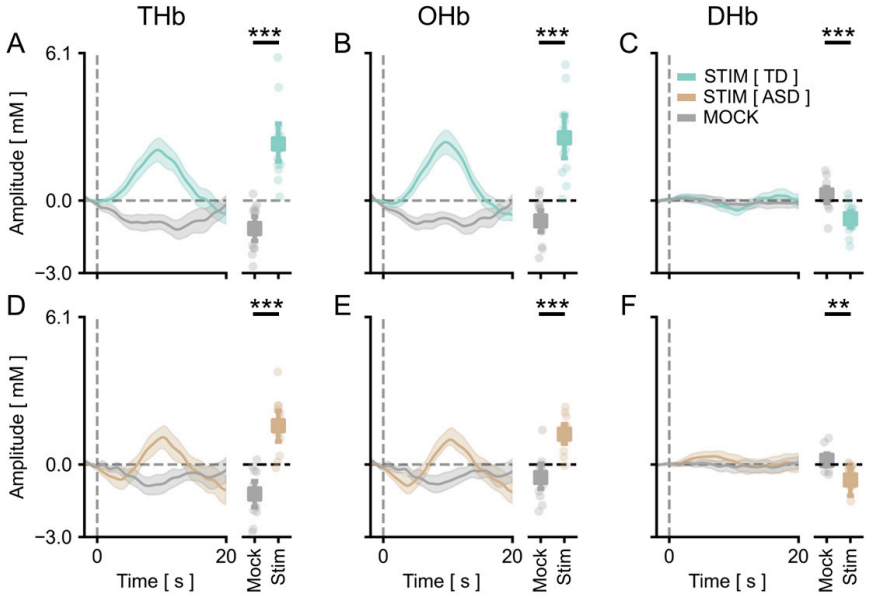

Figure 4.12 vHDR was reliably detected in TD (panel A-C) and fASD (panel D-F) participants: average across channels. In all panels, the values in the y-axis are multiplied by 105. (A–C) On the left, the average time course for THb (**A**), OHb (**B**), and DHb (**C**) in response to the radial stimulus (Stim, green line) or the mock stimulus (Mock, grey line) are shown for TD participants. The plots on the right depict the average peak response to Stim vs. Mock across all the subjects. The stimulus-driven signal was significantly higher with respect to the mock condition for all of the metrics (t-test, $p < 0.001$ for all comparisons). (**D–F**) The same plots as above for the fASD subjects. The stimulus-evoked vHDR was significantly higher with respect to the mock condition (t-test, $p < 0.001$ for THb and OHb, $p < 0.01$ for DHb). The orange lines are the Hb responses to the stimulus in fASD plots. The data are expressed as mean sem. ** $p < 0.01$, *** $p < 0.001$.

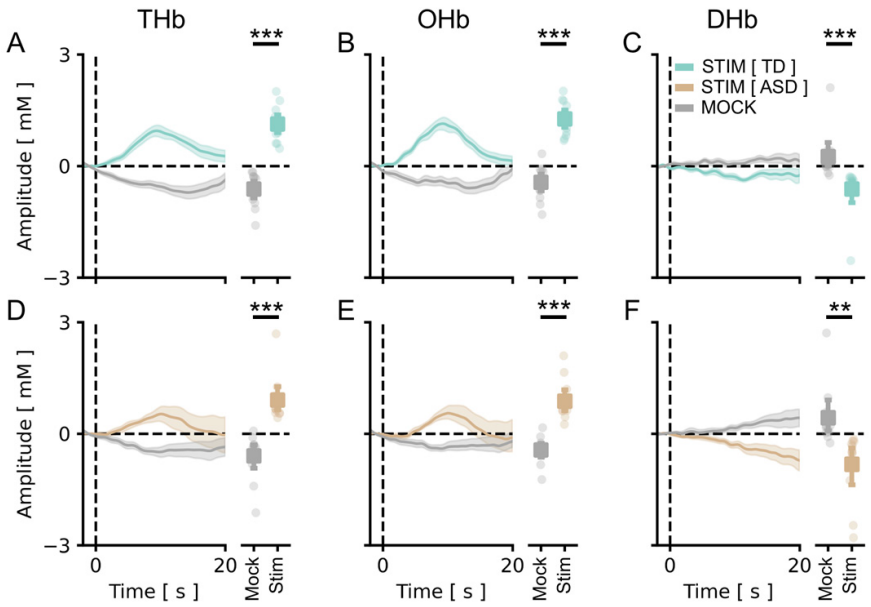

Figure 4.13 vHDR was reliably detected in TD (panel A-C) and fASD (panel D-F) participants: best channel. In all panels, values in the y-axis are multiplied by 104. (A–C) On the left, the average time course for THb (**A**), OHb (**B**), and DHb (**C**) in response to the radial stimulus (Stim, green line) or the mock stimulus (Mock, grey line) are shown for TD participants. Plots on the right depict the average peak response to Stim vs. Mock across all of the subjects. The stimulus-driven signal was significantly higher with respect to the mock condition for all the metrics (t-test, $p < 0.001$ for all comparisons). (**D–F**) The same plots as above, for fASD subjects. The stimulus-evoked vHDR was also significantly higher with respect to the mock condition in this case (t-test, $p < 0.001$ for THb and OHb, $p < 0.01$ for DHb). The orange lines are the Hb responses to the stimulus in fASD plots. The data are expressed as mean sem.** $p < 0.01$, *** $p < 0.001$.

Altogether, these results reinforce the validity of the cartoon-based stimulating procedure to evoke a reliable activation of the visual cortex, showing that this method is also suitable to study visual cortical processing in a clinically relevant population, such as fASD children.

4.2.1.2 The amplitude of visual cortical responses is significantly lower in the fASD cohort

The level of cortical activation in response to the stimulus in TD subjects and fASD children was compared, showing that the average amplitude of cortical changes of OHb concentrations was significantly lower in fASD children with respect to the control age-matched cohort (Fig. 4.14, panel B). No significant difference was detected between the two groups for THb and DHb (Fig. 4.14, panel A,C); only a trend towards reduction can be observed for THb of fASD (Fig. 4.14, panel A). Totally comparable data were found in the analysis of the channel with the highest response (Fig. 4.14, panel D–F). In

contrast, no change in the signal latency was present between the two groups, except for an increased latency of the DHb peak in fASD (Fig. A2 in the Appendix section).

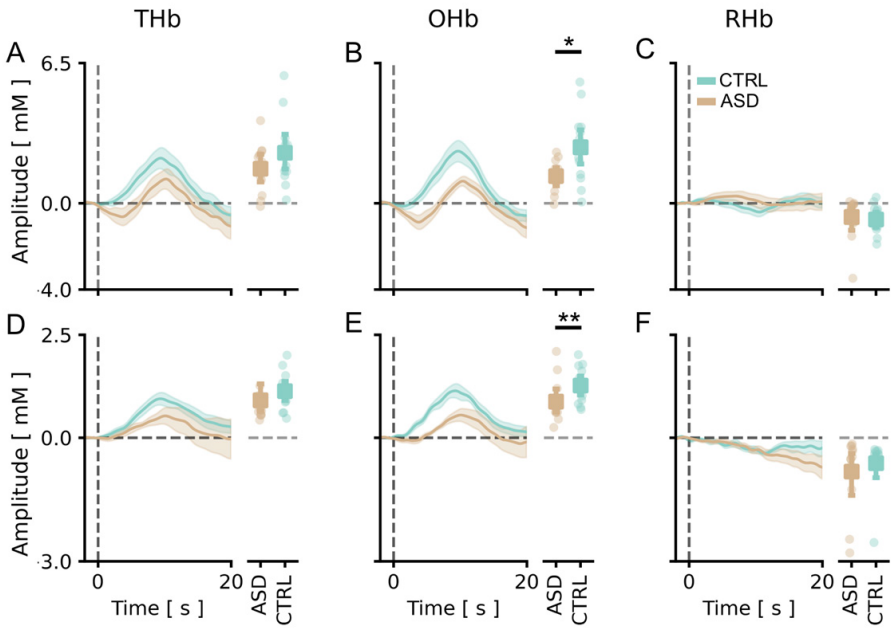

Figure 4.14 Comparison of vHDR between TD and fASD participants. In panels (A–C), values in the y-axis are multiplied by 105; in panels (D–F), values in the y-axis are multiplied by 10^4. (A–C) Average peak responses across all channels of THb (**A**), OHb (**B**) and DHb (**C**) in TD (green lines) and fASD participants (orange lines). An amplitude analysis revealed that the OHb peak was significantly lower in the fASD population than in controls (t-test, $p < 0.05$). (D–F) Peak response of THb (**D**), OHb (**E**) and DHb (**F**) in the best channel for TD (green lines) and fASD subjects (orange lines). The OHb peak was significantly lower in the fASD population than in the controls (t-test, $p < 0.01$). Data are expressed as mean sem. * $p < 0.05$, ** $p < 0.01$.

4.2.1.3 Atypical lateralization of visual responses in the ASD cohort

Since one of the main features of autism neurobiology is the lack of lateralization in brain circuits at a structural and functional level (Floris et al., 2021), visual cortical responses recorded in the right and left hemispheres for all participants has been compared. Notably, average OHb signal detected in the channels mounted upon the right visual cortex of TD children was significantly higher compared to the response recorded through the corresponding optodes of the left hemisphere (Fig. 4.15, panel A,B). Conversely, no interhemispheric difference was detected in the cortical activation of fASD children (Fig. 4.15, panel A,B).

Moreover, the amplitude of signals recorded in the right channels of the TD group was higher with respect to the responses detected in fASD children in the same channels

(Fig. 4.15, panel B). Thus, a laterality index (LI), i.e., the ratio between the average responses in homologous channels between the two hemispheres, was calculated. LI was shifted towards the right hemisphere in TD children, thus strengthening the concept of right predominance in the physiological processing of visual stimuli (Fig. 4.15, panel C). Contrastingly, the occipital cortex of fASD children shows a high variability of LI, with some individuals minimally lateralized, some others left-shifted, and a group of right-lateralized as TD controls (Fig. 4.15, panel A–C). Along the same lines, the distribution of vHDR amplitudes recorded in the two hemispheres highlighted a right bias in TD compared to fASD participants (Fig. 4.15, panel D). Finally, a significant correlation between the dimension of OHb signals in the two hemispheres was found in the control group only (Fig. 4.15, panel E).

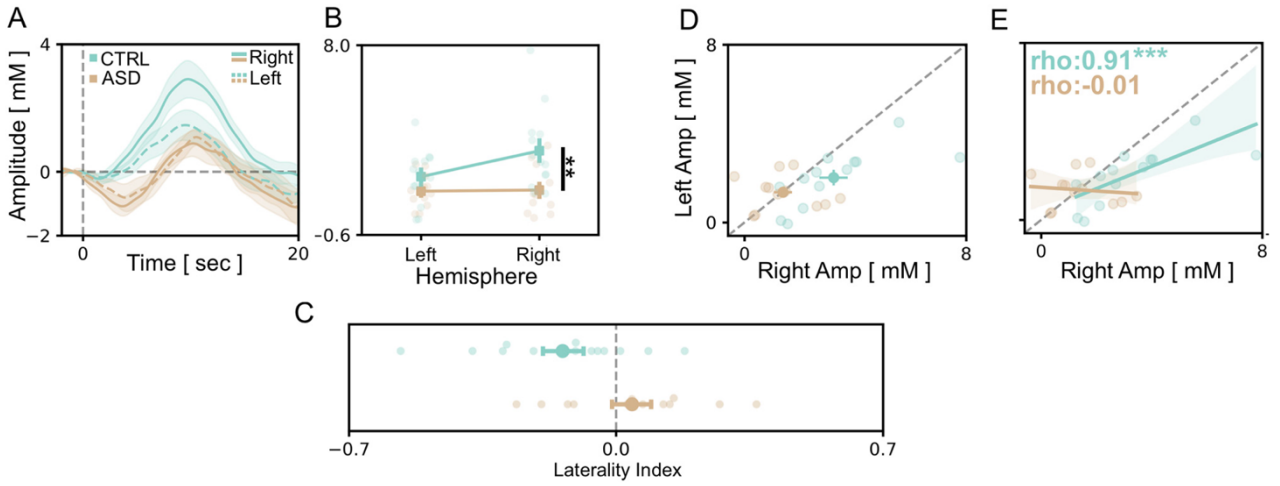

Figure 4.15 Atypical lateralization in visual processing in fASD children. (**A**) Average time course of OHb response in the left (dashed lines) and the right (solid lines) hemispheres for TD (green) and fASD subjects (orange line). The y-axis values are multiplied by 105. (**B**) A comparison between the amplitude of the OHb signal in the left and the right hemisphere showed a significant reduction of right responses in the fASD group (a Two-Way mixed model ANOVA, $p < 0.01$). The y-axis values are multiplied by 105. (**C**) The laterality index, indicating a lower rightward asymmetry of visual processing in fASD subjects compared to TD children (t-test, $p < 0.05$). (**D**) A scatterplot illustrating the distribution of average OHb amplitudes recorded in the right and the left hemisphere in TD and fASD subjects, showing a pronounced rightward bias only in TD children. (**E**) A linear regression analysis shows a significant correlation in the amplitude of average OHb responses evoked in the right and the left hemispheres for TD participants (Spearman correlation, $p < 0.001$). This correlation is absent in ASD patients ($p = 0.965$). The (rho) index indicates the Spearman correlation value. In panels (D,E), the y-axis and x-axis values are multiplied by 105. Levels of statistical significance are shown as ** $p < 0.01$; *** $p < 0.001$.

4.2.1.4 vHDR might be predictive of symptom severity in fASD

Alongside fNIRS recording, a detailed clinical assessment of fASD children was collected, with a range of parent questionnaires and interviews, in addition to direct evaluation (AQ, VABS, ADOS and non-verbal IQ), in order to provide a robust description of the population studied. Thus, the potential correlation between different vHDR metrics and the clinical measures collected was investigated. Although no statistically significant effects were detectable, a trend towards a positive correlation between the amplitude of OHb changes and VABS total score was observed (Fig. A3 in the Appendix section). In addition, the level of response lateralization was negatively correlated to the autistic traits of fASD subjects: indeed, the higher the AQ score revealed by the questionnaire, the lower the extent of the right bias in vHDR (Fig. 4.16, panel A). Despite the low numerosity of the sample, we also observed a trend suggesting a possible correlation between LI and the VABS score, indicating that this vHDR measure might also predict the impairment of adaptive functioning (Fig. 4.16, panel B). No correlation was found with ADOS scores and non-verbal IQ (Fig. 4.16, panel C,D).

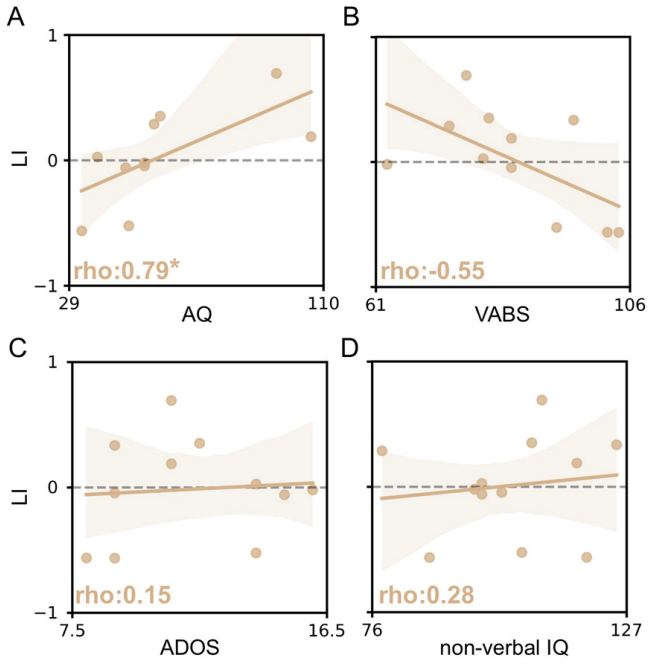

Figure 4.16 Correlation between the laterality index and clinical scores in fASD children. The (rho) index in each plot indicates the Spearman correlation value. The correlation between Laterality Index (LI) and AQ score (**A**), VABS score (**B**), ADOS score (**C**) and non-verbal IQ score (**D**). A positive correlation between variables was detected for the AQ score (Spearman correlation, * $p < 0.05$).

These data were published in June 2023 on Brain Sciences, 13(6): 951 (Scaffei et al., 2023).

4.2.2 Rare metabolic diseases: creatine transporter deficiency (CTD) syndrome

Since feasibility of fNIRS vHDR protocol has been successfully proven in a cohort of fASD, the potential usefulness of this experimental paradigm has been tested in patients with Creatine Transporter Deficiency (CTD), a rare metabolic disorder leading to a complex neurodevelopmental disorder (OMIM 300352). CTD is an X-linked inherited metabolic disorder caused by mutation of SLC6A8 gene that affects cellular uptake of creatine in the brain, thus, causing low cerebral level of this metabolite. CTD presents typically with early intellectual disability, speech and language delay, epilepsy and autistic-like behavior (van De Kamp et al., 2014). Although rare, CTD currently represents the second most common cause of X-linked intellectual disabilities and, thus, a major burden for health care and for patient quality of life. Despite growing knowledge about the natural history of CTD and the role of Cr in energy metabolism, little is known about the brain alterations underlying the impairment of multiple behavioral and cognitive domains in CTD. Since fNIRS signal strongly relies on neurovascular coupling, this technique might reveal a non-canonical HDR due to atypical metabolism and related altered brain activity in CTD.
Thus, a cross-sectional study on the cohort of CTD patients was performed with two specific aims:

- to test the feasibility of fNIRS vHDR procedure previously described in this clinical population;
- to investigate the validity of vHDR as an effective tool to discriminate between CTD and sex/age matched TD peers.

This observational monocentric study was conducted at the IRCCS Stella Maris Foundation (SMF) Hospital (Pisa, Italy) between November 2020 and October 2023. A total of 5 CTD patients who were referred to SMF for clinical visits (mean age 12.2 $\pm$ 6.2 (SD) years) and 5 sex/age-matched control were recruited. Demographics and clinical features of experimental cohort are listed in Tables 5 and 6 in Appendix section. Clinical measures such as linguistic, cognitive and adaptive level, assessed using internationally validated scales/interviews, i.e., Wechsler Scales of Intelligence (Freeman, 2013) and Vineland Adaptive Behavior Scales–II–VABS (Sparrow et al., 2012), were systematically collected for each subject. Clinical relevant data were collected as well, especially regarding frequent CTD comorbidities such as epilepsy. CTD and TD group underwent a single fNIRS recording session according to vHDR protocol previously discussed; only the cartoon-based paradigm was used, repeated twice if

tolerated by the subject in order to increase number of effective trials. Since behavioral phenotype makes a priori this cohort challenging to record, level of compliance during the experiment were qualitatively monitored by the operator, in order to verify mostly fixation of the screen by patients. Data analysis pipeline is consistent with procedure detailed in previous chapter (Material and Methods). Due to the rarity of disorder, patients' age was extremely heterogeneous (age range: 6-22 years). Since vHDR is strongly affected by age in term of range of variability of response amplitude, a division in two more homogeneous group has been done to analyze data (children vs adult group).

4.2.2.1 fNIRS vHDR recording was feasible in all participants and the cartoon-based stimulus evoked reliable responses in CTD patients

The cortical vHDR elicited by a reversing checkerboard pattern merged with an isoluminant commercial cartoon (contrast level 80%) was recorded in the occipital cortex, obtaining a significant activation of the ROIs in response to the stimulus in TD controls and in CTD patients. Grand averages across all participants for Total Hb (THb), Oxygenated Hb, (OHb) and Deoxygenated Hb (DHb) concentration changes in best channel are plotted in Figures 4.17 and 4.18. Preliminary analysis revealed a tendency towards main effect of the checkerboard stimulus (S) with respect to the presentation of the mock stimulus (blank) for all HDR metrics both in control group and, importantly, also in the CTD cohort (Figures 4.17 and 4.18, panel A–B).

4.2.2.1 CTD patients show a higher vHDR compared to age-matched controls

The level of cortical activation in response to the stimulus in TD controls and CTD patients was compared for all the metrics. Preliminary results suggest that CTD patients show a trend towards higher amplitude of hemodynamic response with respect to age-matched controls for OHb and THb metrics. Despite the small sample size, this pattern is more consistent in younger subject (Fig. 4.19, panel A), while a wider variability of vHDR was observed in adult CTD (Fig. 19, panel B).

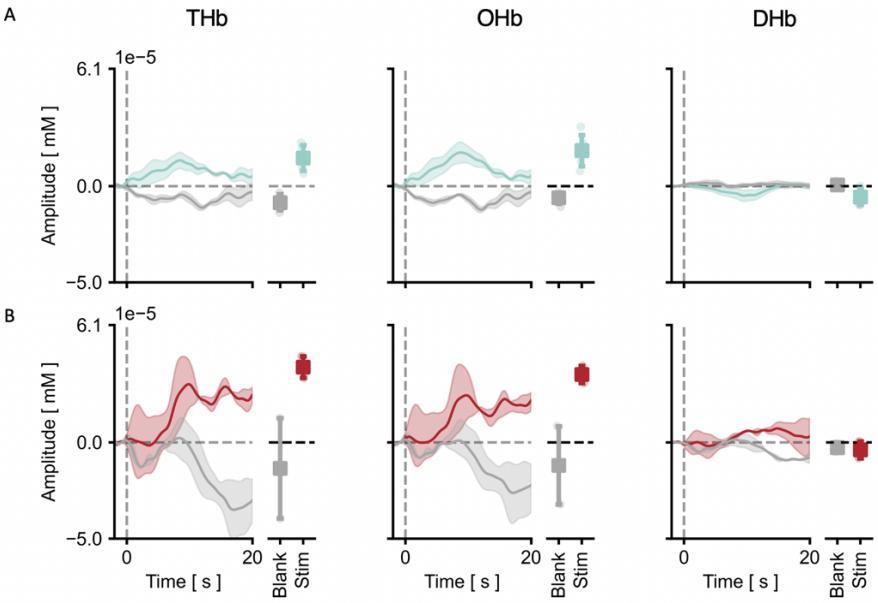

Figure 4.17 vHDR was reliably detected in TD (A row) and CTD (B row) children: best channel. In all panels, values in the y-axis are multiplied by 10^5. (**A**) starting from the left, the average time course for THb, OHb and DHb in response to the radial stimulus (Stim, green line) or the mock stimulus (Mock, grey line) are shown for TD participants. Plots on the right depict the average peak response to Stim vs. Mock across all of the subjects. The stimulus-driven signal was higher with respect to the mock condition for all the metrics (**B**) The same plots as above, for CTD patients. Red lines are the Hb responses to the stimulus in CTD. The stimulus-evoked vHDR was consistently higher with respect to the mock condition for all metrics.

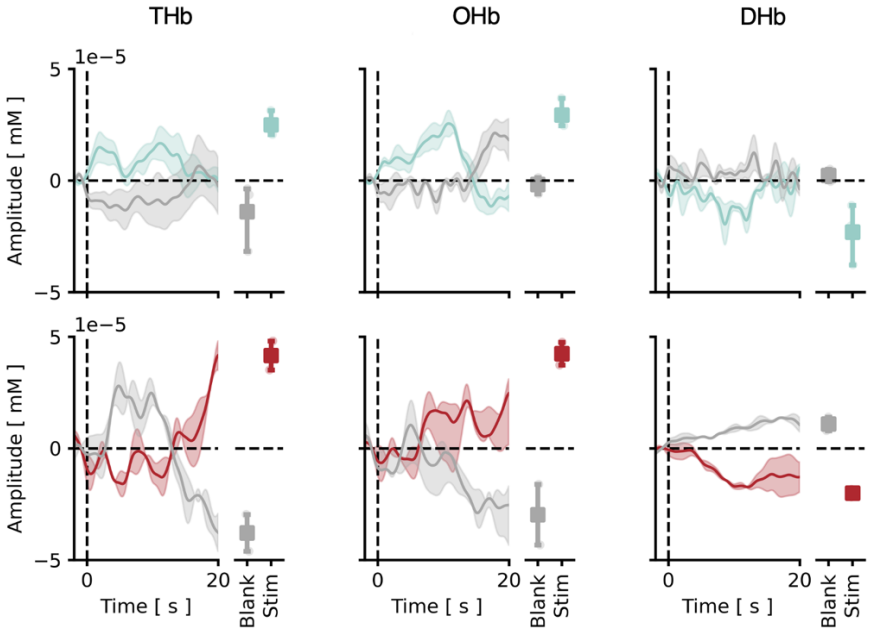

Figure 4.18 vHDR was reliably detected in TD (A row) and CTD (B row) adults: best channel. In all panels, values in the y-axis are multiplied by 10^5. (**A**) starting from the left, the average time course for THb, OHb and DHb in response to the radial stimulus (Stim, green line) or the mock stimulus (Mock, grey line) are shown for TD participants. Plots on the right depict the average peak response to Stim vs. Mock across all of the subjects. The stimulus-driven signal was significantly higher with respect to the mock condition for all the metrics. (**B**) The same plots as above, for CTD patients. The stimulus-evoked vHDR was also consistently higher with respect to the mock condition in this case. The red lines are the Hb responses to the stimulus in CTD plots.

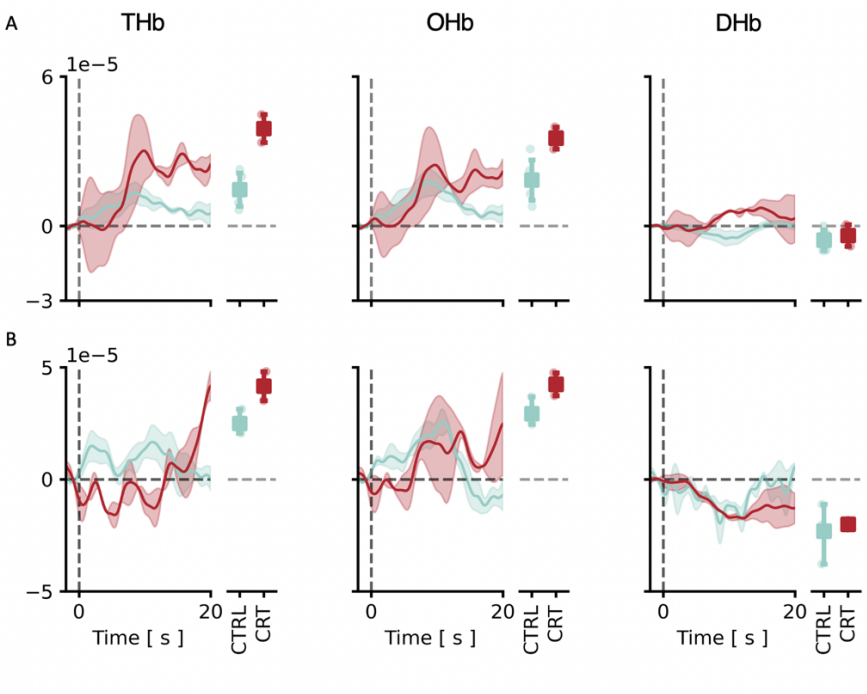

Figure 4.19 Comparison of vHDR between TD and CTD patients: children (A row) and adult (B row) subgroups. In all panels values in the y-axis are multiplied by 10^5. Peak response of THb, OHb and DHb in the best channel for TD (green lines) and CTD subjects (red lines) both for children (A row) and for adults (B row). Small sample size prevents to apply a statistical analysis of vHDR amplitude between the two experimental group. Preliminary results suggest that CTD patients show a trend towards higher amplitude of hemodynamic response with respect to age-matched TD for OHb and THb metrics, more consistently in children subgroup.

5 Discussion

As previously said, compared to other neuroimaging techniques, such as EEG, MEG and fMRI, fNIRS stands out for its portability, non-invasiveness, experimental flexibility to more ecological setting but also reliability of signal and robustness to motion artifacts. FNIRS has already been proven to be reliable in the study of neurodevelopmental trajectories (Vanderwert & Nelson, 2014) and for investigation of sensory perception, both in adults and children. Several studies focused on the characteristics of primary cortical activation, mostly in visual and auditory perception (for the latter see review of van de Rijt et al., 2018). Interestingly, given its methodological advantages, fNIRS could be promising to explore brain activation in response to simple trigger and to study such "low-level" visual cortical activation as prototype a of brain response, since early developmental age too. Previous findings outlined a detectable specific occipital response to simple visual stimulus (like checkerboard pattern) starting from 3 months-old infants (Watanabe et al., 2008) and further exploration on early visual processing came from the study of photostimulation of infants during natural sleep, revealing non-canonical HDR with decreasing of OHb and THb and increasing of DHb (Kusaka et al., 2004). Interestingly, to date, no studies applying a visually-task-related fNIRS design in clinical pediatric samples are reported in literature.

Here, an extensive discussion of results obtained through the investigation of vHDR protocol in several experimental cohorts, ranging from normative population to clinical samples, will be outlined in order to focus both on methodology's strengths and on its limitations to further address.

Starting from the normative population, hemodynamic responses in the occipital cortex while subjects (adults and children) viewed a reversing checkerboard pattern on a gray isoluminant baseline or the same stimulus blended with a commercial animated cartoon was recorded and analyzed. In all participants, the patterned stimulus elicited a significant change of cortical Hb (upwards for THb and OHb, down for DHb) independently from the reference baseline, while no response was detected following blank presentation. Interestingly, the level of occipital cortex activation did not depend on either the movie selected as reference baseline or the baseline contrast, with only a slight reduction of OHb and DHb change in response to the stimulus blended to animated cartoons with respect to the classic RS condition.

To confirm the reliability of this novel visual-evoked metric, a test-retest study has been performed in a cohort of 50 healthy adult subjects at three different timepoints, finding

a good concordance of measurements at group- and individual level analysis. These data reinforce published findings reporting the robustness of visual paradigm in functional neuroimaging studies (Zafar et al., 2015) and in particular in fNIRS studies. Accordingly, previous research addressed the crucial issue of temporal stability of visually-evoked HDR looking at circadian variability (Schroeter et al., 2005) or longer retest interval (Plichta et al., 2006) highlighting the coherence of the measurement, even if with some limitations. Indeed, the results of both reports relied on smaller sample size (respectively 11 and 10 subjects) compared to our cohort. Moreover, Plichta et al. analyzed the reproducibility of fNIRS recordings at three weeks, while the main strength of our study is that we tested longer intersession reliability of fNIRS visual metrics, with a maximum interval period of 2 years. Interestingly, a gender bias in neuroimaging study due to fluctuating hormonal levels of females (Maki & Resnick, 2001) has been raised in some reports as a possible confounding variable in circadian fNIRS HDR measurements (Schroeter et al., 2005) and deserve further investigations. It is worth noting, however, that this variability could be neglected in the studies of X-linked disorders such as CTD because heterozygous females commonly show only a mild phenotype. In addition, the impact of habits, like caffeine or nicotine consumption (Domino et al., 2000; Jacobsen et al., 2002) and sleep deprivation (Soshi et al., 2010), on HDR might be systematically explored in future fNIRS research. Even if our test-retest study on fNIRS vHDR paradigm adds an important contribution to the field, the reliability of this metric needs to be further investigated particularly in developmental cohort.

Altogether, these data demonstrate the validity and reliability of this novel cartoon-based visual stimulation in eliciting cortical responses. Even if our test-retest study was limited to adult subjects and further investigation in developmental cohorts is needed, the high entertaining and ecological value of this approach might be promising for studying cortical function in children with an atypical neurodevelopment, commonly showing a reduced compliance in experimental environments.

The putative correlation between fNIRS HDR metric and some behavioral variables has been tested in the normative population in order to further explore the sensitivity of this procedure of capturing phenotype clinical variability in clinical populations. The magnitude of HDR modulation, and in particular of THb, resulted to be inversely correlated with AQ scores in children: indeed, the higher were the AQ scores of subjects the lower was the amplitude of THb response to visual stimulation. Since levels of THb reflect the relative changes of both OHb and DHb concentrations over the visual cortex,

it could be surmised that the better correlation of THb with AQ scores might be due to a combinatory effect of OHb and DHb variables. Correlations were statistically relevant for AQ total score as well as for AQ "Social Skills" and "Communication" subscales. It is not surprising considering that Social Skills" and "Communication" are the subscales with higher construct validity performance in differentiating individuals with or without ASD (Lundqvist & Lindner, 2017). These data suggest that visually-evoked fNIRS responses are able to capture the dimension of autistic traits in the general young population. Interestingly, these findings are consistent with previous studies showing that cortical activation measured with fNIRS and the performance in visual psychophysics negatively reflected ASD symptom severity (Simmons et al., 2009; Seymour et al., 2019; Noel et al., 2021). A tentative explanation of reduced HDR in children with stronger autistic traits might be found in the difference of perceptual styles in the general population (Turi et al., 2018). Moreover, growing evidence show that ASD is also associated with early abnormalities in multiple sensory processing, fluctuating between hyper- and hypo-sensitivity to sensory stimuli, including visual domain (Simmons et al., 2009). Sensory symptoms usually anticipate signs of social impairment and are correlated with the severity of the disorder, at least in children (Kern et al., 2006).

Based on these premises, the feasibility and validity of vHDR metric has been explored in a cohort of preschooler females with high-functioning ASD.

A first major finding of this study is that in all participants, including fASD children, the patterned stimulus was reliable in eliciting a significant change of cortical Hb with respect to the reference baseline, while no response was detected following the presentation of a mock, grey stimulus. Consistently with previous studies of evoked vHDR (Chen et al, 2019), a rise in total (THb) and oxygenated Hb (OHb), with a parallel negative change in the concentration of reduced Hb (DHb) was observed. Interestingly, the same results emerged from the analysis of the average amplitude of Hb concentration across the 22 channels of the occipital montage, and from the evaluation of the Hb peaks measured in the channel with the highest vHDR within each recording (best channel). These data demonstrate the feasibility of this novel fNIRS procedure in an ASD population with a sub-optimal compliance to structured experimental environments, further establishing the high entertaining value of this cartoon-based stimulation and the ecological merits of the fNIRS tool, according to previous application of technique in the study of atypical brain development (Vanderwert & Nelson, 2014).

Furthermore, the magnitude of vHDR modulation, and in particular of OHb, was significantly different between the two groups studied, with a lower amplitude of OHb response in the visual cortex of fASD subjects compared to TD controls. The selective change of OHb is not surprising considering that this metric is the most commonly used according to literature for investigating evoked HDR in the ASD brain, especially in preschool age (Conti et al., 2022). Moreover, these further findings are also consistent with previous results showing that the activation of cortical circuits negatively reflects autistic traits in TD children, but also ASD symptom severity in the clinical population (Robertson & Baron-Cohen, 2017). These data suggest, for the first time, that vHDR might represent a novel non-invasive, analytical tool to support the diagnostic assessment in females, overcoming the subjective bias intrinsically affecting clinical observations and parental interviews. This provides an important contribution to the field, considering that girls and women with ASD are mostly underserved by diagnostic criteria (Lockwood et al., 2021), making this clinical population strongly underrepresented in autism research (D'Mello et al., 2022). Although previous fNIRS studies on preschoolers suggested the validity of this technique to discriminate between ASD subjects and TD, using both task-evoked and resting-state experimental designs (recently reviewed by Conti et al., 2022), the innovative value of vHDR approach relies on the elevated accessibility of visually evoked recordings requiring a relatively low commitment of tested subjects and allowing an easy extraction of fundamental metrics. Interestingly, reduced vHDR in fASD children might be ascribable to the differences of visual sensory processing repeatedly observed in the ASD brain both at behavioral and neurophysiological level. Indeed, a peculiarity in the visual behavior in terms of abnormal eye contact and the atypical processing of faces as well as distinctive perceptual styles, has been frequently reported in ASD, and emerged during the first year of life (Apicella et al, 2020). Moreover, the well-known preference for focusing on local details vs. the global stimulus configuration in ASD subjects (Van der Hallen et al., 2015) and, thus, the locally centered perception on the screen, might determine a less effective activation of the cortical circuits. In agreement with these data, neuroimaging evidence indicates a low-processing-level origin of autistic sensory traits, showing atypical responses in primary cortices across sensory modalities, increased inter-trial variability of the evoked responses, and cortical ultrastructural abnormalities, involving also occipital areas (Robertson & Baron-Cohen, 2017). Therefore, alterations of the neurochemical and functional architecture of neural circuits detected in the ASD visual cortex might also explain the weaker vHDR in response to the cartoon-based

stimulation, although a possible contribution of top-down attentional modulation of sensory signaling could not be excluded. Moreover, while extensive research has focused on the neural abnormalities underlying ASD, the role of brain vasculature in this disorder remains poorly understood, underestimating neurovascular crosstalk in this population. Recent research addressed extensively this topic (recently revised by Wang et al., 2023) highlighting several possible cerebrovascular deficits in ASD such as impaired cerebral blood supply, altered blood-brain barrier structure and permeability, and atypical neurovascular unit leading to reduced HDR in this population. Interestingly, also at preclinical level, a vascular link in ASD has been established, finding early dysfunction of endothelial cells and impaired endothelium dependent vasodilation in a mouse model of 16p11.2 deletion (Oullette et al., 2020), thus making HDR analysis a promising horizon in the study of ASD pathophysiology.

Furthermore, regarding lateralization of the vHDR, a predominant activation of the right hemisphere in the control group has been noted. A rightward asymmetry of typical visual processing is consistent with the previous literature: indeed, a number of studies combining multiple experimental approaches reported a right hemispheric dominance in typical subjects (Jonas et al., 2014). In contrast, any specific lateralization pattern in the recordings of fASD children has been noted. Accordingly, the loss of brain asymmetry has been widely reported in ASD studies between several developmental age, either in language-related circuits (Conti et al., 2016) or in non-verbal networks, including both higher cognitive domains and primary sensorimotor function (Floris et al., 2021). In the context of the visual system, it is also worth noting that binocular rivalry is weaker in autism (Robertson et al., 2013), and this deficit might be due to the reduced GABAergic action in the visual cortex (Robertson et al., 2016), but also to the reduced strength of interhemispheric connections between the left and right visual cortex (Girault et al., 2022). Despite the fact that previous fNIRS studies focusing on hemispheric asymmetry in ASD individuals reported controversial evidence (Doi et al., 2017) these data reinforce the notion of atypical lateralization in the ASD brain, expanding the assumed topology to other cortical regions than core-symptom ones (Floris et al., 2021). Moreover, since occipital activity is strongly modulated by internal brain states and attentional networks (Reynolds & Chelazzi, 2004), it might be hypothesized that the asymmetry of typical visual dynamics might arise by a leftward bias of attentional systems to the left hemifield (Hougaard et al., 2015), and that the atypical lateralization in the ASD brain reflects at least in part a centralized deficit in domain-general cognitive processes.

Interestingly, the laterality index (LI) is predictive of the severity of autistic traits measured by AQ children's version. Indeed, the higher were the AQ scores of subjects, the weaker was the rightward asymmetry of visual responses, suggesting that this measure within an individual is able to capture the level of autistic phenotype in the fASD cohort. In contrast, no significant correlation between LI and the other observational scales evaluating the extent of the behavioral impairment of subjects was found. This is likely due to the low numerosity of the experimental sample, but also to the relatively high homogeneity of the cohort and, thus, the respective clinical measures' output range. Indeed, the fASD cohort in study presented a restricted variation range in the ADOS-2 comparison score, suggesting a similar inter-subject symptom severity. Moreover, a correlation trend between LI and the VABS score was detected, even if not statistically significant, suggesting that VABS scores were more capable than non-verbal IQ scores in terms of highlighting the functional impairment of fASD children according to previous studies on this topic (Alvares et al., 2020).

Finally, in order to verify the feasibility and the validity of vHDR paradigm in a rare metabolic disorder where neurovascular coupling might be primarily affected, visually-evoked fNIRS recordings were collected in a sample of patients with CTD.

Preliminary results suggest that CTD patients show a higher amplitude of hemodynamic response with respect to age-matched controls. Due to the rarity of disease, clinical studies in this cohort are to date sparse, focused primarily on clinical phenotyping rather than to more specific functional neuroimaging approach, making, thus, comparison with literature evidence less informative. Interestingly, preclinical studies have been recently boosted by the availability of several murine model of the disease such as whole-body rodent models (Skelton et al., 2011; Baroncelli et al., 2014) and brain- and cell-specific conditional mouse models (Baroncelli et al., 2016; Udobi et al., 2018). Indeed, animal models are an excellent tool to dissect the disease pathogenetic mechanisms and drive the preclinical development of therapeutics. Moreover, murine models available to date have proven to reliably reproduce cognitive, behavioral and functional phenotypes of CTD disease (for an updated review on this topic see Ghirardini et al., 2021), thus allowing the study of quantitative biomarkers of translational value for monitoring disease progression and response to therapeutics in preclinical, and hopefully clinical, studies. Indeed, available clinical data from neurochemical assays and from Magnetic Resonance Spectroscopy, even if crucial to lead diagnostic pipeline, are not directly correlated with symptoms' severity and, thus, are less informative on disease progression (van de Kamp et al., 2014). In this field interesting evidence came from

the recent study of novel functional biomarkers for CTD, suitable both in rodents and in patients, such as alteration of cortical oscillations at EEG spectral analysis or, notably, atypical neurovascular coupling detected by cortical optical imaging (Mazziotti et al., 2020). Since the main sources of fNIRS signal superimpose with those of optical imaging (Zepeda et al., 2004), our preliminary findings in CTD patients support previous evidence from preclinical model of disease highlighting an increased HDR in response to cortical activation in the knockout mouse (Mazziotti et al., 2020). At the neurobiological level it is not surprising that the forced metabolic phenotype and the augmented oxidative stress in CTD brain (Giusti et al., 2019) could dynamically upregulate the cerebral blood flow stimulating vasodilation (Watts et al., 2018). However, the low numerosity of the sample still prevented to draw final conclusion about the validity of fNIRS biomarker in CTD and to explore correlation between HDR and symptom severity.

However, some limitations need to be discussed and addressed in future studies.

First, the small size of clinical samples recruited to date, both for fASD and CTD cohort. Moreover, although fASD cohort was very well characterized, we need to acknowledge that it represents a very selected sample in terms of both gender and developmental quotient, thus preventing too much generalization of our results to the whole ASD population. Further studies in a larger cohort of subjects, including children with a larger range of symptom severity, are needed in order to assess the sensitivity and the specificity of this analytical tool. In particular, it would also be interesting to study the amplitude of fNIRS signals in age-matched males in order to explore potential influence of gender-dimension on the results. Indeed, ASD females have previously shown a distinctive pattern of resting state EEG activity compared to ASD males (Neuhaus et al., 2021), suggesting that research concerning biomarkers needs to consider the moderation of the biological sex. Small sample size is a crucial issue for rare genetic disease like CTD. In this scenario, natural history studies are increasingly needed, also to detect reliable phenotyping biomarkers and monitor disease progression. To overcome this point a multisite European study (EJP RD JTC 2022, IMAGINER PROJECT) involving Stella Maris Institute and the Hospital of Lyon has been already started with expected total recruitment of 20 subjects and shared outcome measures, like clinical and neurobehavioral endpoints.

Secondly, a more extensive evaluation of reliability of vHDR metric will be assessed in order to explore, and potentially minimize, confounding effect linked to circadian variability of signal and arousal level of subject.

6 Conclusion and future perspectives

To conclude, the potential feasibility and validity of a novel visually-evoked fNIRS paradigm (vHDR) has been systematically explored and confirmed in several experimental cohorts, including both TD population and, importantly, clinical samples of under investigated NDDs. Indeed, sensitivity and reliability of vHDR metrics have been robustly assessed in a large cohort of adults and children, even optimizing the methodology in order to ensure maximal compliance to experimental setting for its application in NDDs. Then, two pilot cross-sectional studies were conducted on a cohort of fASD preschoolers and on a group of CTD patients comparing fNIRS response with age and sex-matched controls. Results suggested significantly different vHDR dynamics in these two clinical cohorts, reinforcing the concept of fNIRS as a valuable biomarker in NDDs. Even if both these pilot studies included small samples of subjects, evidence about the potential of vHDR fNIRS protocol set the background for the replication of this novel paradigm in a larger cohort of children with NDDs. Indeed, for ASD group, a wider sample, including children with a larger range of symptom severity as well as male subjects is needed to further assess the accuracy of this analytical tool and the validity of LI measurement. Similarly, for CTD cohort further evidence is needed to establish the robustness of this biomarker, hopefully reproducing results in a multisite study, thus overcoming difficulty in recruitment due to the rarity of disease. Moreover, in order to establish the specificity of vHDR as a valuable biomarker the comparison with other complex NDDs, such as X-linked ID (in particular Fragile X Syndrome) or Angelman Syndrome is ongoing.

Thus, the use of vHDR as a biomarker of brain function might significantly optimize clinical diagnosis and follow-up of under investigated NDDs, thus, providing, a reliable and unbiased measure to track atypical developmental trajectories and supporting, in combination with behavioral testing, the evaluation of tailored intervention strategies and the study of drug efficacy in clinical trials.

Abbreviations

AD: Alzheimer Disease
ADHD: Attention Deficit / Hyperactivity Disorder
ADOS: Autism Diagnostic Observation Schedule
AQ: Autism Questionnaire
ASD: Autism Spectrum Disorder
BD: block design
BOLD: Blood Oxygen Level Dependent
CC: cartoon chosen condition
CF: cartoon fixed condition
CTD: Creatine Transporter Deficiency
CW: Continuous Wave
DHb: deoxygenated hemoglobin
EEG: electroencephalography
EF: executive functions
ERD: Event-related Design
fASD: females with autism spectrum disorder
FC: functional connectivity
fMRI: functional magnetic resonance imaging
fNIRS: functional Near Infrared Spectroscopy
FR: Frequency-Resolved
HDR: hemodynamic response
HRF: hemodynamic Response Function
ID: Intellectual Disability
IFG: inferior frontal gyrus
IOS: intrinsic optical signal
IPL: inferior parietal lobe
M1: primary motor cortex
MCI: Mild cognitive impairment
MEG: magnetoencephalography
MNS: mirror neuron system
MS: Multiple Sclerosis
NDDs: Neurodevelopmental Disorders
OHb: oxygenated hemoglobin

PD: Parkinson Disease

PFC: prefrontal cortex

ROI: region of interest

RS: Radial Stimulus condition

SNR: signal to noise ratio

STS: superior temporal sulcus

TBI: Traumatic Brain Injury

TD: typical development

THb: total hemoglobin

TPJ: temporoparietal junction

TR: Time-Resolved

VABS: Vineland Adaptive Behavior Scales

VFT: verbal fluency task

vHDR: visual-evoked hemodynamic response

WM: working memory

WSCT: Wisconsin Card Sorting Test

Appendix

ID	Age	Gender	Head	Cap	AQ	AQ_S	AQ_C	AQ_A	AQ_D	AQ_I	CF	CC
A1	29	F	57	56	11	0	1	7	1	2	The Lion King Walt Disney Pictures,1994	The Sword in the Stone Walt Disney Productions,1963
A2	36	F	56	56	17	0	0	6	9	2	The Powerpuff Girls special episode "Twas the Fight Before Christmas", Cartoon Network Studios, 2003	The Aristocats Walt Disney Productions,1970
A3	35	M	57	56	17	3	3	5	0	6	Kung Fu Panda DreamWorks Animation, 2008	Aladdin Walt Disney Pictures,1992
A4	28	F	57	56	3	0	0	1	1	1	Peppa Pig "Hide-and-seek", "Fly the kite", "Polly parrot" episodes, Entertainment One, 2004	The Simpsons S29E02 "Springfield Splendor", 20th Television, 2017
A5	25	F	57	56	19	4	1	7	6	1	The Lion King Walt Disney Pictures,1994	101 Dalmatians Walt Disney Productions,1961
A6	35	F	57	56	15	4	2	4	5	0	The Powerpuff Girls special episode "Twas the Fight Before Christmas", Cartoon Network Studios, 2003	The Simpsons S29E02 "Springfield Splendor", 20th Television, 2017
A7	29	F	57	56	11	0	2	5	3	1	Peppa Pig "Hide-and-seek", "Fly the kite", "Polly parrot" episodes, Entertainment One, 2004	101 Dalmatians Walt Disney Productions,1961
A8	30	F	56	56	13	0	2	4	5	2	Kung Fu Panda DreamWorks Animation, 2008	Wall-E Disney-Pixar, 2008
A9	29	F	59	56	7	1	1	3	2	0	The Lion King Walt Disney Pictures,1994	The Sword in the Stone Walt Disney Productions,1963
A10	29	M	58	56	12	1	1	5	4	1	The Powerpuff Girls special episode "Twas the Fight Before Christmas", Cartoon Network Studios, 2003	Aladdin Walt Disney Pictures,1992
A11	27	M	56.5	56	17	2	3	5	4	3	Peppa Pig "Hide-and-seek", "Fly the kite", "Polly parrot" episodes, Entertainment One, 2004	101 Dalmatians Walt Disney Productions,1961
A12	30	F	56	56	9	1	2	2	3	1	Kung Fu Panda DreamWorks Animation, 2008	Inside out Disney-Pixar, 2015
A13	29	M	57	56	14	1	3	6	2	2	The Lion King Walt Disney Pictures,1994	Wall-E Disney-Pixar, 2008
A14	36	M	58.5	56	19	2	2	5	7	3	The Powerpuff Girls special episode "Twas the Fight Before Christmas", Cartoon Network Studios, 2003	101 Dalmatians Walt Disney Productions,1961
A15	36	M	57	56	16	4	1	4	5	2	Peppa Pig	Aladdin Walt Disney Pictures,1992

											"Hide-and-seek", "Fly the kite", "Polly parrot" episodes, Entertainment One, 2004	
A16	29	F	54	56	22	1	3	9	7	2	Kung Fu Panda DreamWorks Animation, 2008	The Rescuers Walt Disney Productions,1977
A17	28	F	58.5	56	7	1	0	2	1	3	The Lion King Walt Disney Pictures,1994	101 Dalmatians Walt Disney Productions,1961
A18	29	F	55	56	12	0	1	5	5	1	The Powerpuff Girls special episode "Twas the Fight Before Christmas", Cartoon Network Studios, 2003	Peter Pan Walt Disney Productions,1953
A19	34	M	60	56	19	6	2	5	5	1	Peppa Pig "Hide-and-seek", "Fly the kite", "Polly parrot" episodes, Entertainment One, 2004	SpongeBob S12E01 "FarmerBob", Nickelodeon Animation Studio, 2018
A20	26	M	58	56	10	3	0	5	2	0	Kung Fu Panda DreamWorks Animation, 2008	Inside out Disney-Pixar, 2015
A21	36	F	56	56	15	1	2	2	7	3	The Lion King Walt Disney Pictures,1994	Inside out Disney-Pixar, 2015
A22	30	M	60.5	56	19	1	2	6	4	6	The Lion King Walt Disney Pictures,1994	The Sword in the Stone Walt Disney Productions,1963
A23	26	F	56	56	12	0	1	5	6	0	The Powerpuff Girls special episode "Twas the Fight Before Christmas", Cartoon Network Studios, 2003	Wall-E Disney-Pixar, 2008
A24	32	M	60.5	56	7	0	1	5	0	1	Kung Fu Panda DreamWorks Animation, 2008	Aladdin Walt Disney Pictures,1992
A25	34	F	56	56	10	1	1	4	2	2	The Lion King Walt Disney Pictures1994	Beauty and the Beast Walt Disney Pictures,1994
A26	29	F	57	56	32	6	5	8	9	4	The Powerpuff Girls special episode "Twas the Fight Before Christmas", Cartoon Network Studios, 2003	The Aristocats Walt Disney Productions,1970
A27	31	M	59	56	32	10	6	6	2	8	Kung Fu Panda DreamWorks Animation, 2008	The Simpsons S29E02 "Springfield Splendor", 20th Television, 2017
A28	39	F	57	56	21	2	4	4	8	3	Peppa Pig "Hide-and-seek", "Fly the kite", "Polly parrot" episodes, Entertainment One, 2004	Lady Oscar, "A Funeral Bell Tolls in the Twilight" episode, Discotek Media, 1980
A29	29	M	57	56	6	0	0	2	2	2	The Lion King Walt Disney Pictures,1994	The Simpsons S29E02 "Springfield Splendor", 20th Television, 2017
A30	29	M	57	56	17	3	2	5	6	1	The Powerpuff Girls special episode "Twas the Fight Before Christmas", Cartoon Network Studios, 2003	The Sword in the Stone Walt Disney Productions,1963
A31	36	M	59.5	56	12	0	0	6	4	2	Peppa Pig "Hide-and-seek", "Fly the kite", "Polly parrot" episodes, Entertainment One, 2004	The Sword in the Stone Walt Disney Productions,1963
A32	27	M	59	56	12	1	2	5	2	2	The Powerpuff Girls special episode "Twas the Fight Before Christmas", Cartoon Network Studios, 2003	The Aristocats Walt Disney Productions,1970
A33	40	M	57	56	13	2	3	1	6	1	Peppa Pig "Hide-and-seek", "Fly the kite", "Polly parrot" episodes, Entertainment One, 2004	The Simpsons S29E02 "Springfield Splendor", 20th Television, 2017

A34	31	F	55	56	15	1	1	4	4	5	Kung Fu Panda DreamWorks Animation, 2008	The Sword in the Stone Walt Disney Productions,1963
A35	27	M	56.5	56	19	6	2	4	7	0	The Lion King Walt Disney Pictures,1994	Frozen Walt Disney Pictures, 2013
A36	30	M	58	56	14	5	1	3	4	1	The Powerpuff Girls special episode "Twas the Fight Before Christmas", Cartoon Network Studios, 2003	Wall-E Disney-Pixar, 2008
A37	25	M	58	56	29	6	8	6	7	2	Peppa Pig "Hide-and-seek", "Fly the kite", "Polly parrot" episodes, Entertainment One, 2004	Wall-E Disney-Pixar, 2008
A38	31	M	57.5	56	10	0	1	5	3	1	Kung Fu Panda DreamWorks Animation, 2008	Inside out Disney-Pixar, 2015
A39	38	M	57.5	56	25	2	4	6	8	5	The Lion King Walt Disney Pictures,1994	The Sword in the Stone Walt Disney Productions,1963
A40	33	F	57.5	56	13	0	0	3	10	0	The Powerpuff Girls special episode "Twas the Fight Before Christmas", Cartoon Network Studios, 2003	Beauty and the Beast Walt Disney Pictures,1994

Table 1 Demographic characteristics of adult subjects.
Age (years), gender, head circumference (head, cm), cap size (cm), total AQ score (AQ), AQ subscale scores (AQ_S, AQ_C, AQ_A, AQ_D, AQ_I) and the movies used for visual stimulation (CF and CC according to the experimental protocol) are listed for each participant. For movies, production company, release date, and episode title are indicated as well.

ID	Age	Gender	Head	Cap	AQ	AQ-S	AQ_	AQ_	AQ_	AQ_I	C1 (L1 and H1)	C2 (L2)
B1	5	M	51.5	52	21	1	4	7	5	4	Uncle Grandpa S3E04 "Uncle Easter", Cartoon Network Studios, 2016	Teen Titans Go! To the Movies Warner Bros. Animation, 2018
B2	5	M	51	52	32	6	7	3	10	6	Wile E. Coyote & Road Runner "Coyote falls", ""Fur of flying" and "Rabid rider" episodes, Acme Corporation, 2010	ABCs song Little Baby Bum - Nursery Rhymes & Kids Songs youtube channel, 2014
B3	13	M	57	56	26	6	3	4	7	6	The Simpsons S29E02 "Springfield Splendor", 20th Television, 2017	Futurama, S7E01 "The Bots and the Bees", Comedy Central, 2012
B4	12	M	56	56	44	8	8	11	13	4	The Incredibles Disney-Pixar, 2004	Wile E. Coyote & Road Runner "Coyote falls", ""Fur of flying" and "Rabid rider" episodes, Acme Corporation, 2010
B5	12	M	56	56	25	1	4	10	0	10	Big Hero 6 Walt Disney Pictures, 2014	The Amazing World of Gumball S01E01 "The DVD", Cartoon Network Development Studio Europe, 2011
B6	6	F	53	52	17	1	1	4	8	3	Inside out Disney-Pixar, 2015	Floopaloo S1E22 "Squirrel for a Day", Marc du Pontavice, 2012
B7	7	M	53	52	24	2	4	1	12	5	The Chipmunks "Bye, George" episode, DIC Entertainment, 1989	Floopaloo S1E22 "Squirrel for a Day", Marc du Pontavice, 2012
B8	4	F	50.5	52	38	6	11	7	11	3	Frozen Walt Disney Pictures, 2013	Bolt Walt Disney Pictures, 2008
B9	4	M	52	52	42	1	11	11	13	6	Spider-Man: Into the Spider-Verse Columbia Pictures, 2018	Bolt Walt Disney Pictures, 2008
B10	4	M	51	52	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Cars Walt Disney Pictures, 2006	Thomas & Friends "Diesel and the duckling" episode, Mattel, 2019
B11	9	F	54	56	28	2	6	3	13	4	The Simpsons S29E02 "Springfield Splendor", 20th Television, 2017	Zig & Sharko S01E28 "Moby Zig", Xilam Animation, 2010
B12	8	M	54.5	56	26	4	5	5	4	8	Beauty and the Beast Walt Disney Pictures,1994	Ice Age Blue Sky Studios, 2002
B13	4	M	53	56	44	11	9	8	9	7	Finding Nemo Disney-Pixar 2003	101 Dalmatians Walt Disney Productions,1961
B14	6	M	53	52	36	5	2	3	19	7	Trolls DreamWorks Animation, 2016	Curious George S1E03 "Zeros to Donuts", Universal Anim. Studios, 2006
B15	7	M	51.5	52	49	12	8	10	9	10	Cars Walt Disney Pictures, 2006	Pup Academy S1E02"Tell Us About Your Human Day", AirBud Ent, 2019
B16	10	F	55	56	48	9	9	10	10	10	Frozen Walt Disney Pictures, 2013	Descendants Disney Channel Original Productions, 2015
B17	10	M	54	56	40	9	5	7	13	6	The Simpsons S29E02 "Springfield Splendor", 20th Television, 2017	Uncle Grandpa S3E04 "Uncle Easter", Cartoon Network Studios, 2016
B18	4	F	49.5	52	19	2	3	5	6	3	Frozen Walt Disney Pictures, 2013	Bing S1E06 "Smoothie",Tandem Films&Digitales Studios, 2014
B19	7	M	54	56	19	2	3	5	6	3	Ranger Rob S1E12 "Big Stink in Big Sky Park" Nelvana Enterprises Inc, 2016	Arex&Vastatore "Casket of fear" episode from YouTube Channel, 2021

Table 2 Demographic characteristics of children. Age (years), gender, head circumference (head, cm), cap size (cm), total AQ score (AQ), AQ subscale scores (AQ_S, AQ_C, AQ_A, AQ_D, AQ_I), and the movies used for visual stimulation (C1 and C2 according to the experimental protocol) are listed for each participant. For movies, production company, release date, and episode title are indicated as well.

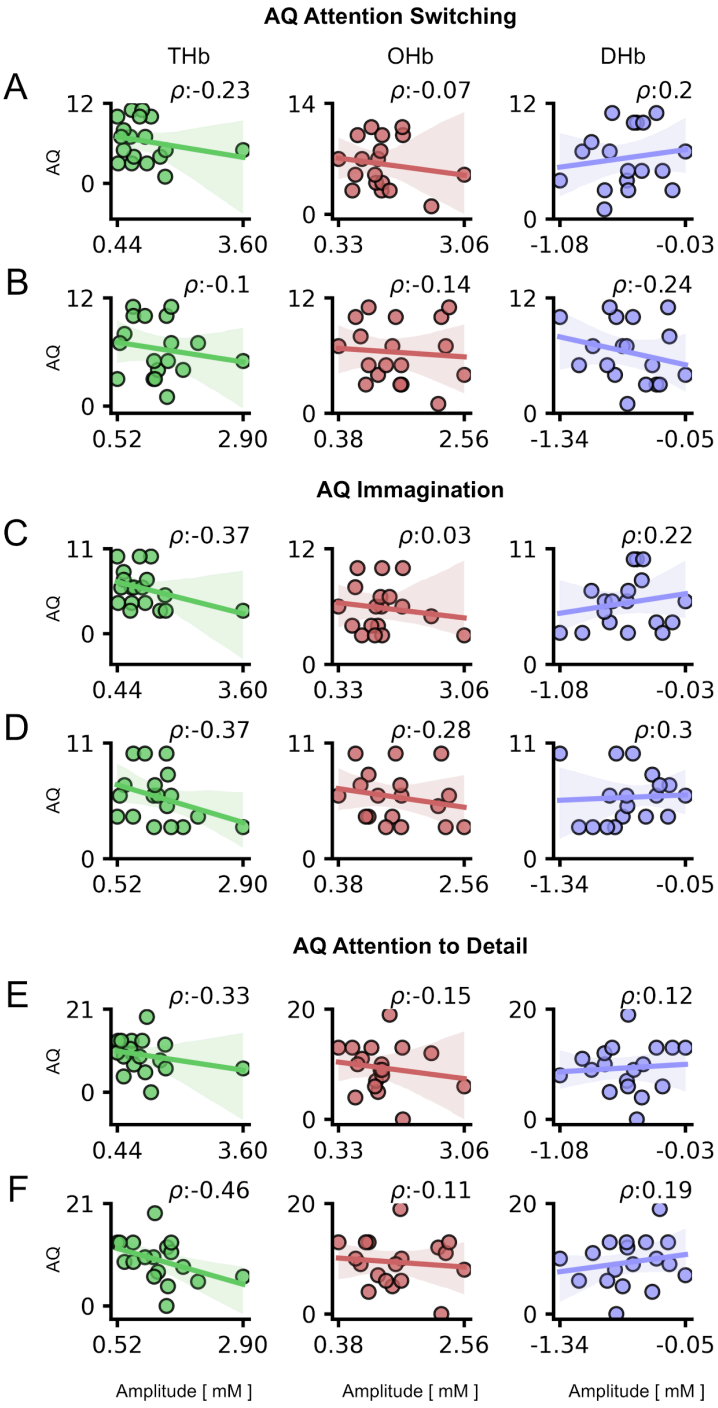

Figure A1 Correlation between HDR and AQ subscales in children. For all panels, values in the x-axis are multiplied for 10^4. The ρ (rho) index in each plot indicates the Spearman correlation value. No significant correlation was present Between THb, OHb, DHb metrics and Attention Switching (A,B), Imagination (C,D) and Attention to Detail (E,F) AQ subscales.

ID	Age	Gender	Head	Cap size	Cartoon selected
A1	4	F	49	52	Frozen Walt Disney Pictures, 2013
A2	5	F	50.5	52	The Little Mermaid Walt Disney Pictures,1989
A3	6	F	53	54	The Chipmunks, S7E04 "Bye, George" MWS and DIC Entertainment
A4	5	F	50.5	52	The Lion King Walt Disney Pictures,1994
A5	4	F	50.5	52	The Chipmunks, S7E04 "Bye, George" MWS and DIC Entertainment
A6	6	F	53	54	SpongeBob, S12E01 "FarmerBob" Nickelodeon Animation
A7	3	F	51	52	The Little Mermaid Walt Disney Pictures,1989
A8	4	F	49.5	52	Frozen Walt Disney Pictures, 2013
A9	6	F	53	54	Inside out Disney-Pixar, 2015
A10	6	F	53	54	The Little Mermaid Walt Disney Pictures,1989
A11	4	F	41.5	54	Frozen Walt Disney Pictures, 2013
A12	6	F	50.5	54	Frozen Walt Disney Pictures, 2013
A13	3	F	50	52	The Aristocats Walt Disney Productions,1970

Table 3 Demographic data of the control group. Age (years), gender, head circumference (cm), cap size (cm), and the cartoon used for visual stimulation are listed for each participant (for movies, the production company, release date, and episode title are indicated as well).

ID	Age	Gender	Head	Cap size	Cartoon selected	ADOS TOT	ADOS comp	AQ tot	Nv IQ	Cognitive Scale	VABS tot
B1	4	F	51	52	Frozen Walt Disney Pictures, 2013	16	7	53	96	GMDS III	63
B2	5	F	50.5	52	Frozen Walt Disney Pictures, 2013	n.a.	n.a.	56	78	Leiter III	74
B3	6	F	53	54	Bing, S1E06 "Smoothie" Tandem Films e Digitales Studios, 2014	14	5	38	98	WPPSI III	80
B4	4	F	51	52	Frozen Walt Disney Pictures, 2013	9	5	n.a.	125	Leiter III	96
B5	3	F	48.5	52	Bing, S1E06 "Smoothie" Tandem Films e Digitales Studios, 2014	14	5	48	106	WPPSI III	93
B6	6	F	55	56	Uncle Grandpa, S3E04 "Uncle Easter" Cartoon Network Studios, 2016	12	7	58	108	WISC IV	81
B7	5	F	50	52	The Chipmunks, S7E04 "Bye, George" MWS and DIC Entertainment	9	6	96	87	GMDS III	87
B8	6	F	52	52	Frozen Walt Disney Pictures, 2013	11	6	106	117	WPPSI III	85
B9	5	F	51.5	52	Bing, S1E06 "Smoothie" Tandem Films e Digitales Studios, 2014	9	5	53	102	WPPSI III	85
B10	4	F	52	52	Frozen Walt Disney Pictures, 2013	15	5	47	98	GMDS III	n.a.
B11	3	F	53	52	Curious George S1E03 "Zeros to Donuts", Universal Animation Studios, 2006	8	4	33	119	WPPSI III	102
B12	3	F	48.5	52	Masha and the Bear S1E26 "Home" Improvement, Animaccord Animation Studio	11	5	95	110	Leiter III	77

Table 4 Demographic data of the fASD group. Age, gender, head circumference, cap size, and the animated cartoon used for vHDR are listed for each participant. Moreover, clinical variables collected, such as the ADOS total and comparative score, the total AQ score, non-verbal IQ scores, and the VABS total score are listed for each subject. For non-verbal IQ scores, the psychometric scale used is specified: total score for the Leiter III scale; performance score for the WPPSI III scale; Perceptual Reasoning Index for the WISC IV scale; the mean score between "Foundations of learning" and "Eye and hand coordination" areas was used for GMDS III developmental assessment.

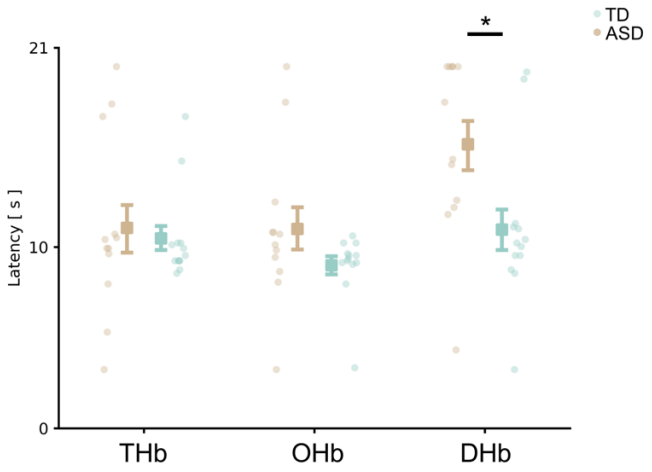

Figure A2 Latency of vHDR in TD and fASD subjects. No differences of vHDR latencies were detected for average THb and OHb peaks, whereas a significantly higher latency of DHb was found in fASD children (Two-Way ANOVA, $p < 0.05$).

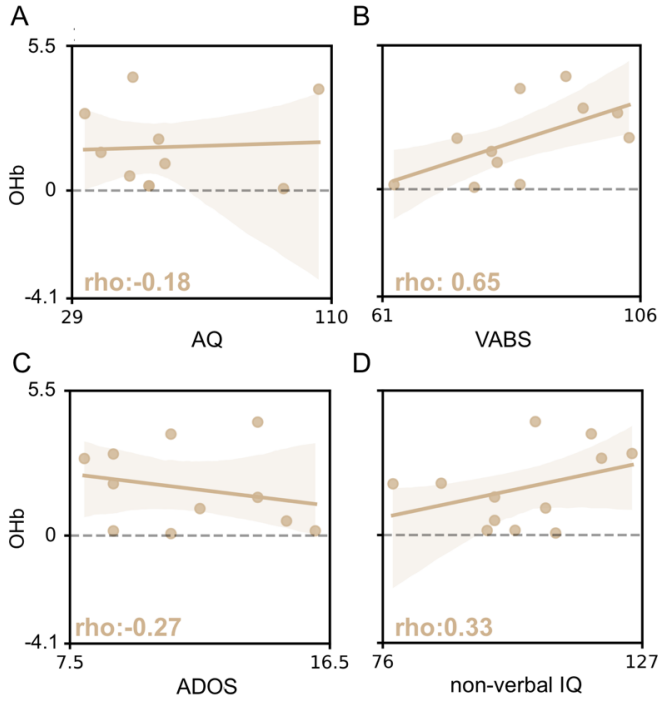

Figure A3 Correlation between the amplitude of OHb signals and clinical scores in fASD. The ρ (rho) index in each plot indicates the Spearman correlation value. Correlation between average OHb amplitude and AQ score (**A**)**,** VABS score (**B**), ADOS score (**C**) and non-verbal IQ score (**D**). No significant correlation between variables was detected. The y-axis values are multiplied by 10^5.

Patient ID	Family pedigree	Age at diagnosis (y)	Mutations	MRI	MRS (Brain Cr)	CR/CRN urine	Treatment
CT1	Mother and sister heterozygous	5	c.1631C>T	Normal	Reduced	1.83	Arginine 300 mg/kg/die+valproate Ch 750 mg/die
CT2	*De novo*	5.6	c.1255-14_1255-24del	Normal	Reduced	2.24	Arginine 300 mg/Kg/die os
CT3	Mother and sister heterozygous	5.5	c.757 G>C	Normal	Reduced	3.08	Arginine 300 mg/Kg/die os
CT4	*De novo*	18	n.a.	Normal	n.a.	n.a.	None
CT5	Mother heterozygous	4	c.1271_1291dup	White matter hyperintensity in the posterior regions; thin corpus callosum	Reduced	4,99	Creatine 210 mg/kg/die Arginine 300 mg/kg/die Glicine 150 mg/kg/die Carbamazepine 300mg/die Risperidone 1.25 mg/die

Table 5 Demographics of CTD cohort: genetic, biochemical and neuroradiological data

Patient ID	Last visit and fNIRS test	Seizures, age at onset	Neurological signs	Cognitive level	Adaptive level	Language	Behavior
CT1	22 y	Generalized T-C drug – reistent 5 yrs	Mild Hypotonia	Moderate ID	Moderate deficit	Severe deficit	Hyperactivity, impulsiveness
CT2	11 y	No epilepsy	/	Mild ID	Mild deficit	Mild deficit	ADHD
CT3	17 y	Isolate febrile convulsions, 3 years	Clumsiness	Moderate ID	Mild deficit	Severe deficit with oromotor dispraxia	Autistic-like behavior
CT4	18 y	No epilepsy	/	Mild ID	Borderline adaptive functionning	Mild deficit	Autistic-like behavior
CT5	6 y	Generalized T-C febrile convulsions 2.5y	Stereotypic movements	Severe ID	Severe deficit	Absent language (isolate words)	Autistic-like behavior, hyperactivity and inattention

Table 6 Demographics of CTD cohort: clinical data

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Ringraziamenti

Vorrei ringraziare tutte le persone che hanno contributo a questo lavoro e alla nascita del Laboratorio.
Un grazie speciale a Laura Baroncelli, non solo per tutte le cose che mi ha insegnato in questi anni, ma soprattutto per la generosità d'animo mostrata.
Ringrazio la Prof.ssa Battini, alla quale vanno il mio affetto e la mia incondizionata stima.
Grazie a Francesco e alla mia famiglia che mi hanno supportato con amore anche in questo percorso.