# Clinical Study Protocol

## *Repurposing mirtazapine in Rett syndrome: a multicentric open label Phase II study*

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| Protocol Code | MirtaRett |
| Investigational Product Name/Number | Mirtazapine |
| Short title | Repurposing mirtazapine in Rett syndrome: a multicentric open label Phase II study |
| Sponsor Name and Legal Registered Address | University of Trieste<br>Dipartimento di Scienze della Vita (DSV)<br>Via via Edoardo Weiss, 2 34128Trieste (ITALY)<br>Telephone: +39 040 558 8724 (Office) –<br>+39 040 558 2443 (Lab)<br>Fax: +39 040 558 2445 |
| EU CT No. | 2024-515411-21-00 |
| Version Number | 2.0 |
| Date | 20/12/2024 |

## Statement of confidentiality

*Information in this protocol and accompanying documents contains privileged or confidential information that is the property of University of Trieste. It is understood that the information will not be used, divulged, or published without prior written consent of University of Trieste, except to the extent such disclosure is required by applicable laws and regulations.*

## Protocol Signature Sheet and Acknowledgement

The **Università degli Studi di Trieste** and the Sponsor representative have discussed this protocol version and agreed that it contains all necessary details for carrying out the trial. The investigations will be performed as by protocol except in the case of medical emergency.

Problems related to this trial should be referred to the **Scientific Coordinator**, **Prof Enrico Tongiorgi**.

The Scientific Coodinator agrees to conduct and supervise the clinical study.

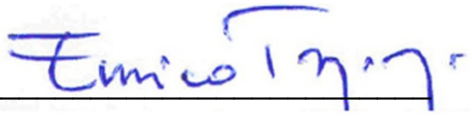
The sponsor hereby confirms that all investigators and institutions participating in this clinical trial agree to permit clinical trial-related monitoring, audits, and regulatory inspections. This includes providing direct access to source data and documents.

Telephone: +39 040 558 8724 (Office) –

+39 040 558 2443 (Lab)

Fax: +39 040 558 2445

**Sponsor Signature:**

Signature:  Date: 16/07/2024

Prof. Enrico Tongiorgi, Scientific Coordinator

## Study Personnel

### SPONSOR

Contact Name: Prof Enrico Tongiorgi

Position: Scientific Coordinator MirtaRett

Address: Università degli Studi di Trieste, Via L. Giorgieri 5, Building Q - 34127 Trieste - ITALY

Tel: +39 040 558 8724 (Office) - +39 040 558 2443 (Lab)

Fax: +39 040 52445

Email: tongi@units.it

### PHARMACOVIGILANCE

Contact Name: CVBF Safety Management Team

Address: Consorzio per Valutazioni Biologiche e Farmacologiche (CVBF), Viale Cesare Battisti 17, Pavia, Italy

Tel.: +39 373 8530614

Fax: +39 080 9909321

Email: pharmacovigilance@cvbf.net

### INVESTIGATIONAL SITES

The full list of investigational sites will be kept in the Trial Master File and reported below:

- Partner 1. Centro Epilessia – Unità Neurologia Pediatrica, ASST Ospedale Santi Carlo Paolo - Dipartimento Scienze della Salute, Università di Milano.
- Partner 2. Unità di Neuropsichiatria Infantile, IRCCS, Istituto Giannina Gaslini, Genova.
- Partner 3. Unità di Pediatria, Dipartimento della Donna e dei Bambini – Policlinico S. M. alle Scotte. Siena.
- Partner 4. Dipartimento di Patologia Umana dell'Adulto e dell'Età Evolutiva "Gaetano Barresi", Policlinico Universitario "G. Martino, University of Messina. Messina.

# 1. Synopsis

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| **Study Title** | Repurposing **Mirtazapine** in Rett syndrome: a multicentre, open label, single- arm, phase II study (**MirtaRett Study**). |
| **Study code**<br>**EU CT NUMBER** | MirtaRett<br>2024-515411-21-00 |
| **Study drug** | Mirtazapine (MTZ), a noradrenergic and specific serotonergic tetracyclic antidepressant (NaSSA) with an excellent safety profile. |
| **Background** | Rett Syndrome (RTT) is a rare disease caused by a specific genetic mutation in the MECP2 gene located on the female X chromosome and therefore particularly affects female subjects. It is characterised by an altered development of the nervous system and therefore it leads to an intellectual deficit.<br>Some of the common symptoms associated with the Rett syndrome include:<br>a) Learning problems and delayed development of motor and cognitive skills<br>b) Loss of already acquired language skills<br>c) Involuntary hand movements such as twisting, tapping or pushing, and hand-washing type movements<br>d) Coordination and balancing problems<br>e) Difficulties in verbal and non-verbal communication<br>f) Repetitive behaviour, such as hand flapping or biting<br>g) Breathing problems, such as sleep apnoea or hypoventilation<br>h) Gastrointestinal problems, such as constipation or gastro-oesophageal reflux<br>i) Alterations in mood and social behaviour, such as |

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| | irritability, anxiety or estrangement<br>j) Sleep problems, such as insomnia or restless sleep.<br><br>Due to the fact that Rett syndrome is a progressive degenerative condition, symptoms may get worse over time and may be different from person to person. Particularly in childhood, the syndrome results in severe disability that makes impossible the active participation in physical activities and affects the ability of conception.<br><br>**Background data for dose selection**<br><br>Antidepressant drugs are typically administered using a scalar dosing approach, starting with low doses and gradually increasing them. This method allows for monitoring individual responses to the medication, as higher initial doses can lead to more severe side effects. Antidepressants generally take 2-4 weeks to show clinical effects, necessitating a careful titration schedule. For instance, the typical starting dose of mirtazapine in adults is around 15 mg/day, with a maximum of 50 mg/day, while studies have also examined its use in specific populations, such as those with major depressive disorder or respiratory issues. In children and adolescents, lower doses are recommended due to potential side effects like gastrointestinal issues and self-harm, which are particularly relevant for patients with Rett syndrome. |
| **Hypothesis and preliminary results** | The reduced in monoamine levels (serotonin, noradrenaline and dopamine) observed in RTT, suggested the possibility to use antidepressant treatments such as MTZ, to improve symptoms. RTT Mirtazapine (MTZ) is a tetracyclic noradrenergic and serotonergic specific antidepressant (NaSSA) with a favourable safety profile. In young male MeCP2-null mice, MTZ induced a marked improvement in cardiac and respiratory rhythm deficits, restored glutamatergic and GABAergic transmission and fully recovered microcephaly; in female MeCP2 heterozygous mice, it reduced sensory hypersensitivity and improved motor |

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| | skills.<br><br>In adult patients, MTZ promoted improved sleep, mood, hand stereotypies, social interactions and aggression. Therefore, considering these preliminary results in humans, we hypothesise that MTZ could be beneficial in RTT children. |
| **Study design** | We propose a multicentre, open label, single-arm, phase II study to evaluate safety and efficacy of MTZ in the treatment of mood, sleep quality, motor symptoms in particular hand control, in Rett syndrome children and adults. Other outcomes such as autonomic function, behavioural, caregiver burden and overall clinical severity will be examined along with neuroplasticity and metabolism biomarkers. |
| **Number of study participants** | Subjects included in the study are female patients with a diagnosis of RTT, confirmed by mutation of the MECP2 gene which is responsible for the development of the disease, and who meet the inclusion/exclusion criteria of the study.<br><br>The total number of participating patients is 54, aged between 5 and 40 years, divided into three groups of 18 patients each (5-10 years, 11-17 years and 18-40 years). |
| **Sample size justification** | The minimum sample size (15 patients per age group) has been estimated considering the rarity of the disease under study and the preliminary results obtained in an open label test of mirtazapine in adult RTT patients.<br><br>Primary endpoint: Motor-Behavior Assessment Scale (MBAS) change from Baseline.<br><br>Summary Statement: A sample size of 15 achieves 90.5% power to detect a difference of 18.0% between the null hypothesis (H0) of MBAS change from Baseline mean of -20.0% and the alternative hypothesis (Ha) of MBAS change from Baseline mean of -38.0% with an estimated standard deviation of 19.8% and with a significance level (alpha) of 0.05 using a two-sided one- |

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| | sample t-test.<br><br>Assuming approximately 15% of drop-out rate, 54 patients are expected to be enrolled. |
| **Inclusion criteria** | Patients can only be included in the study if all the following conditions are satisfied:<br><br>1. Female aged 5 to 39 years inclusive, at the time of signing the informed consent.<br>2. Girls of childbearing age negative to pregnancy test.<br>3. Body weight > 10 kg.<br>4. Diagnosis of RTT based on consensus clinical criteria (Neul, 2010) and a confirmed mutation in MECP2 gene.<br>5. Breathing dysfunction (at least one of the following): period apnoea, intermittent hyperventilation, breath holding spells, air swallowing, forced expulsion of air and /or saliva.<br>6. Ten episodes or more/day of breathing dysfunction during wakefulness in the week prior to the screening visit (parents report).<br>7. Stable medication regimen for 4 weeks prior to beginning the study (if receiving services - physical, occupational, or speech therapy - subjects must be on a stable regimen of these services for 3 months prior to beginning the study).<br>8. Female patients of childbearing potential must use a highly effective contraceptive method such as combined hormonal contraception (containing estrogen and progestin) associated with ovulation suppression (oral, intravaginal, transdermal); progestin-only hormonal contraception associated with ovulation suppression (oral, injectable, implantable); intrauterine device; hormone-releasing intrauterine system. Sexual abstinence is considered a highly effective contraceptive method if it aligns with the individual's usual lifestyle.<br>9. Written consent signed by parent/legal guardian/representative prior to screening visit |

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| | 10. Patient is cooperative, willing to complete the study, and capable of doing so with assistance of a caregiver.<br>11. Caregiver is able to understand the instructions and fully participate. |
| **Exclusion criteria** | The female patients cannot be included in the study if they meet one of the following exclusion criteria:<br>1. they are participating in another investigational clinical trial;<br>2. Hypersensitivity to MTZ;<br>3. Clinically significant (as determined by the investigator) cardiovascular, respiratory, gastrointestinal, renal, hepatic, haematological pathologies or other pathologies, in addition to those directly related to RTT. In particular, patients with the following parameters will be excluded: leucocyte count is < 4000/mm2; neutrophil count is < 2000/mm3; hyponatremia (< 125 mmol/L); renal dysfunction (creatinine > 2 X ULN), hepatic dysfunction (AST, ALT, bilirubin > 2 X ULN); or if severe diabetes mellitus is present;<br>4. QTcF interval at ECG > 450 msec;<br>5. Scheduled surgery during the study;<br>6. Severe diabetes mellitus (hyperglycaemia with values above 250/300 mg/dL);<br>7. Pregnancy, breastfeeding.<br>8. Evidence of clinically significant malnutrition with BMI (or BMI) (kg/m2) < 13.<br>9. Patients who manifested prior suicidal ideation. |
| **Population assessment** | A total of three populations will be used for treatments and analyses. Study participants who have satisfied the enrolment criteria will be classified in the designated population and will only be included in analyses for which they have available data.<br>Data analysis will be performed using the following analysis sets:<br>• The **Enrolled** (ENR) population will consist of all |

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| | patients who are enrolled into the study (i.e., signed ICF and met the eligibility criteria) and may or may not receive the study treatment.<br>• The **Intent to Treat** (ITT) population will consist of all patients who received at least one dose of study medication and have at least Baseline (Day 0) and End of Treatment (week 24) assessments with primary efficacy data. The ITT population will be used to present efficacy data.<br>• The **Per-Protocol** (PP) set would include all ITT patients who met all inclusion/exclusion criteria liable to affect the primary assessment and did not present major protocol deviations. The detailed reasons for excluding patients from the PP set will be fully defined and documented before database lock. The PP population will be used to present efficacy data.<br>• The **Safety** population will consist of all patients who received at least one dose of study medication. The Safety population will be used to present the demographic, baseline data and all safety data.<br>The ITT will be the primary efficacy analysis set; the same analysis will be additionally performed on the PP set: any discrepancy between results obtained on both analysis sets will be discussed in the Clinical Study Report. All population sets for analysis will be discussed during data review meeting and authorization of subject's inclusion/exclusion from each analysis set will be defined prior to the database lock.<br>Patient disposition and withdrawals, including count and percentage of patients screened, screen failures, completed the study, discontinued the study, patient meets newly developed or not previously recognized and reason for discontinuation will be presented for the ENR population. Number of patients in each population, including reasons for exclusion, will be presented for |

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| | ENR population.<br><br>Subjects who withdraw prematurely can be part of any population (Safety, ITT or PP) depending on the individual follow-up characteristics and according to population derivation. Data collected on study patients up to the time of withdrawal must remain in the trial database. |
| **Drug administration** | From the day of the baseline visit (referred to as T0) to day 14 of the study, all enrolled subjects will start taking the drug with minimum dosage (Dose level 1) per age group:<br>- 3.75 mg/per day (0.25 ml) oral solution for group 5-10 years;<br>- oral solution of 7.5 mg/day (0.50 ml) for group 11-17 years old<br>- oral solution of 15 mg/day (1 ml) for group 18-40 years.<br>Afterwards from Day 15 until Week 24, the optimal dosage to be achieved (Dose level 2) will be:<br>- oral solution of7.5 mg/ per day (0.50 ml) for group 5-10 years;<br>- 15 mg/ oral solution per day (1 ml) for group 11-17 years;<br><br>- oral solution of 30 mg/ per day (2 ml) for group 18-40 years. |
| **Primary objective** | The study aims to evaluate the efficacy of MTZ to induce improving changes in social behaviour,orofacial/respiratory and motor/physical area and to assess the safety of MTZ.<br><br>This study will provide information on what symptoms will MTZ be able to correct, depending on the age and severity of the subject. |
| **Secondary objectives** | 1. To assess the effectiveness of MTZ in improving anxiety, depression and mood.<br>2. To assess the effectiveness of MTZ in improving sleep quality.<br>3. To assess the effectiveness of MTZ in improving hand function. |

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| | 4. To assess the improvement or worsening of the subject's general clinical condition over time.<br>5. To assess the effectiveness of the drug in reducing the severity and number of symptoms associated with Rett syndrome.<br>6. To assess the effectiveness in improving lung function. This will be assessed by measuring thoracic and abdominal respiratory movements, airflow, arterial oxyhaemoglobin saturation (HbSaO2), heart frequency and posture.<br>7. To assess the improvement in parental/carer stress. The rating scale used will be the Parenting Stress Index (PSI-SF).<br>8. To assess the improvement in the levels of neurotrophic factors involved in the regulation of mood and cognition. |
| **Primary endpoint** | The rating scale used is the Motor-Behavior Assessment Scale (MBAS).<br><br>The drug will be considered effective if the treatment decreases the Motor-Behavior Assessment Scale (MBAS) score (maximum score=68) by at least 8.5 points (12.5%) compared to baseline.<br><br>Adverse events (AEs) and serious adverse events (SAEs), both related and non-related. |
| **Secondary endpoints** | 1. The drug will be considered effective if the treatment reduces the overall Anxiety, Depression, and Mood Scale (ADAMS) score (maximum score=84) by at least 10.6 points (12.5%) and reduces the overall Rett Syndrome Behaviour Questionnaire (RSBQ) score by at least 11.25 points (12.5%).<br><br>2. The drug will be considered effective if the treatment improves the measurements obtained from the medical devices for remote sensing (Youcare Smart T-shirt and the Actigraphy), and the Sleep disturbances scale for children (SDSC) scores by at least 20% compared to the initial value. |

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| | 3. The drug will be considered effective if the Purposeful Hand Function scale (PHF) score increases at least 2 points above the baseline score.<br><br>4. The drug will be considered effective if the Clinical Global Impression of Change scale (CGI-C) has a score decrease at least 1 point.<br><br>5. The drug will be considered effective if the Rett Syndrome Severity Scale (RCSS) scores decrease at least 3%, i.e. by 2 points.<br><br>6. The assessment will be performed through measurements of thoracic and abdominal respiratory movements, airflow, arterial oxyhaemoglobin saturation (HbSaO2), heart rate and posture.<br><br>7. The rating scale used will be the Parenting Stress Index (PSI-SF). An improvement in parental stress will be considered as such, if the Parenting Stress Index (PSI-SF) shows a reduction of at least 20%.<br><br>8. The effectiveness of the drug will be assessed by considering a 20% increase from baseline in serum levels of the biomarkers BDNF, GDNF and PDGF. |
| **Study duration** | Total duration of the study: 10 months<br>Duration of the screening phase: 3 months<br>Duration of treatment phase: 6 months<br>Duration of follow-up: 1 month |
| **Visits scheduled** | Number of visits or contacts scheduled:<br>1. Screening (from Day -90 to Day 0)<br>2. T0 Baseline (Day 0),<br>3. I phone follow-up (2 weeks ± 1 day),<br>4. T1 (4 weeks ± 1 week from T0),<br>5. II phone follow-up (8 weeks ± 1 week from T0),<br>6. T2 (12 weeks ± 1 week from T0), |

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| | 7. III phone follow-up (16 weeks ± 1 week from T0),<br>8. T3 (24 weeks ± 1 week from T0)<br>9. T4 follow-up visit (1 months ± 1 week from the last drug administration) |
| **Study timeline in details** | **1. Screening** (Day -90, Day 0): the screening phase will be carried out in the three months leading up to the baseline visit. Subjects will be considered for the study if fulfilling inclusion criteria. During the Screening visit, the following procedures/assessments will be performed and data collected:<br>a) Obtaining signed informed consent (prior to any study procedure)<br>b) Collection of demographic data<br>c) Medical history<br>d) Clinical assessment<br>e) Neurological assessment<br>f) Pregnancy test (only for post-puberty patients)<br>g) Body measurement for ordering the Youcare Smart T-shirt wearable device that will be delivered at the hospital at T0.<br>h) Delivery of the wrist actigraph (Motionlogger) to the patient.<br><br>**2. T0 Baseline (Day 0):**<br>During the day 0 visit, the following procedures/assessments will be performed, and data collected:<br>a) Confirmation of study inclusion/exclusion criteria<br>b) Vital signs (body temperature, heart rate, respiratory rate and blood pressure)<br>c) Standard 12-lead electrocardiogram<br>d) Clinical assessment<br>e) Neurological assessment<br>f) Laboratory tests (haematology, biochemistry, TSH, metabolism biomarkers, serum prolactin, ACTH, and cortisol)<br>g) Pregnancy tests (only for post-puberty patients) |

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| **Study timeline in details** | h) Biomarkers of neuronal plasticity (BDNF, GDNF PDGF)<br>i) Anthropometric assessment<br>j) Polysomnographic assessment (only for patients enrolled in the Genova and Milano centres)<br>k) Delivery of wrist actigraph (Motionlogger) to the patient.<br>l) Delivery of the Youcare Smart T-shirt wearable device and instructions on its use. Cardiorespiratory and nighttime sleep quality evaluation is performed at home for 24 hours, during the first week after the T0 visit<br>m) Completion/evaluation of clinical questionnaires (RCSS, MBAS, ADAMS, RSBQ, PHF, SDSC, CGI-C and PSI-SF<br>n) Dispensing of study drug according to minimum dosage per age group<br>o) Data collection/evaluation of adverse events (AEs) - reported by the patient/caregiver or observed by the investigator<br>p) Data collection/evaluation of serious adverse events (SAEs) - reported by the patient/caregiver or observed by the investigator<br>q) Collection of concomitant medication data<br>r) Handing over of the patient's diary in which the caregiver should record the dispensing data of the experimental drug and any undesirable effects and concomitant drugs.<br><br>**3. I phone follow-up (Week 2)**<br><br>The investigator will contact the family to obtain information about the data in the patient diary in order to assess the occurrence of any drug-related undesirable effects, and to check the tolerability of the experimental drug dose administered. During the call, the following data will be collected:<br><br>a) Adverse events (AEs) - reported by the patient/caregiver;<br><br>b) Serious adverse events (SAEs) - reported by the |

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| **Study timeline in details** | patient/caregiver;<br>c) Concomitant medications taken by the patient;<br>d) Compliance with experimental drug;<br>e) Guidance on increasing the dosage of experimental drug to be administered to patients.<br>**4. T1 (4 weeks ± 1 week from T0**)<br>During the visit T1, the following procedures/assessments will be performed and data collected:<br>a) Vital signs (body temperature, heart rate, respiratory rate and blood pressure)<br>b) Standard 12-lead electrocardiogram<br>c) Clinical assessment<br>d) Neurological assessment<br>e) Laboratory tests (haematology, biochemistry, TSH, metabolism biomarkers, serum prolactin, ACTH, and cortisol)<br>f) Delivery of wrist actigraph (Motionlogger) to the patient.<br>g) Assessment of cardiorespiratory parameters and nighttime sleep quality performed at home for 24h within the first week after the T1 visit (YouCare T-shirt)<br>h) Completion/assessment of clinical questionnaires (RCSS, MBAS, ADAMS, RSBQ, PHF, SDSC, CGI-C and PSI-SF)<br>i) Dispensing of study drug (if necessary) and accounting of drug used by patients<br>(j) Data collection/evaluation of adverse events (AEs) - reported by the patient/caregiver or observed by the investigator<br>(k) Data collection/assessment of serious adverse events (SAEs) - reported by the patient/caregiver or |

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| **Study timeline in details** | observed by the investigator<br>l) Collection of concomitant medication data<br>m) Evaluation of patient diary<br>n) Pregnancy test (only for post-puberty patients).<br><br>**5. II phone follow-up (8 weeks ± 1 week after T0)**<br>The investigator will contact the family to obtain information about the data recorded in the patient diary in order to assess the occurrence of any drug-related undesirable effects, and to check the tolerability of the dose of experimental drug administered. During the call, the following data will be collected:<br>a) Adverse events (AEs) - reported by the patient/caregiver<br>b) Serious adverse events (SAEs) - reported by the patient/caregiver<br>c) Concomitant medications taken by the patient<br>d) Compliance with experimental drug<br><br>**6. T2 (12 weeks ± 1 week from T0)**<br>During the VISIT T3, the following procedures/assessments will be performed and data collected:<br>a) Vital signs (body temperature, heart rate, respiratory rate and blood pressure)<br>b) Standard 12-lead electrocardiogram<br>c) Clinical assessment<br>d) Neurological assessment<br>e) Laboratory tests (haematology, biochemistry, TSH, metabolism biomarkers, serum prolactin, ACTH, and cortisol)<br>f) Biomarkers of neuronal plasticity (BDNF, GDNF |

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| **Study timeline in details** | PDGF)<br>g) Delivery of wrist actigraph (Motionlogger) to the patient.<br>h) Assessment of cardiorespiratory parameters and nighttime sleep quality performed for 24h at home within first week after the T2 visit (YouCare T-shirt)<br>i) Completion/assessment of clinical questionnaires (RCSS, MBAS, ADAMS, RSBQ, PHF, SDSC, CGI-C and PSI-SF)<br>j) Dispensing of study drug (if necessary) and accounting of drug used by patients<br>k) Data collection/evaluation of adverse events (AEs) - reported by the patient/caregiver or observed by the investigator<br>l) Data collection/assessment of serious adverse events (SAEs) - reported by the patient/caregiver or observed by the investigator<br>m) Collection of concomitant medication data<br>n) Evaluation of patient diary<br>o) Pregnancy test (only for post-puberty patients).<br>**7. III phone follow-up (16 weeks ± 1 week after T0)**<br>The investigator will contact the family to obtain information about the data in the patient diary in order to assess the onset of any drug-related adverse reactions, and to check the tolerability of the administered dose of the experimental drug. During the call, the following data will be collected:<br>a) Adverse events (AEs) - reported by the patient/caregiver<br>b) Serious adverse events (SAEs) - reported by the patient/caregiver<br>c) Concomitant medications taken by the patient |

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| **Study timeline in details** | d) Compliance with the experimental drug<br><br>**8. T3 (24 weeks ± 1 week from T0): End of Treatment.**<br><br>During the visit T3 the following clinical procedures/assessments will be performed and the following data will be collected:<br><br>a) Vital signs (body temperature, heart rate, respiratory rate and blood pressure)<br><br>b) Standard 12-lead electrocardiogram<br><br>c) Clinical assessment<br><br>d) Neurological assessment<br><br>e) Laboratory tests (haematology, biochemistry, TSH, metabolism biomarkers, serum prolactin, ACTH, and cortisol)<br><br>f) Pregnancy tests (only for post-puberty patients)<br><br>g) Biomarkers of neuronal plasticity (BDNF, GDNF PDGF)<br><br>h) Delivery of wrist actigraph (Motionlogger) to the patient.<br><br>i) Polysomnographic evaluation (only for patients enrolled in the Genova and Milano centres)<br><br>j) Assessment of cardiorespiratory parameters and nighttime sleep quality performed for 24h at home within first week after the 3 days T3 visit (YouCare T-shirt)<br><br>k) Clinical questionnaires completion/assessment (RCSS, MBAS, ADAMS, RSBQ, PHF, SDSC, CGI-C and PSI-SF)<br><br>l) Collection of study drug bottles (used, unused or partially used)<br><br>m) Data collection/evaluation of adverse events (AEs) - reported by the patient/caregiver or observed by the |

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| **Study timeline in details** | investigator<br>n) Data collection/assessment of serious adverse events (SAEs) - reported by the patient/caregiver or observed by the investigator<br>o) Data collection about concomitant medication<br>p) Evaluation of patient diary<br>q) Return of study treatment kits and You Care T-shirt<br>**9. T4 Follow-up visit (1 month ± 1 week since last drug administration)**<br>During the follow-up visit, the following procedures/assessments will be performed and data collected:<br>a) Vital signs (body temperature, heart rate, respiratory rate and blood pressure)<br>b) Standard 12-lead electrocardiogram<br>c) Clinical assessment<br>d) Neurological assessment<br>k) Completion/evaluation of CGI-C questionnaire<br>m) Data collection/evaluation of adverse events (AEs) - reported by the patient/caregiver or observed by the investigator<br>n) Data collection/evaluation of serious adverse events (SAEs) - reported by the patient/caregiver or observed by the investigator<br>o) Data collection concomitant medication |
| **Trial interventions** | Study medication (3.75 mg, 7.5 mg, 15 mg, 30 mg of MTZ oral solution) will be given once daily at bedtime. During the first 14 days of the treatment period, the oral solution of the active drug at Dose Level 1 will be used to achieve the planned target daily dose, according to age (3.75 mg for 5-10 yrs, 7.5 mg for 11-17 yrs and 15 mg > 18 yrs, from day 1 to 14). From Day 15 to the end of |

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| **Trial interventions** | week 24, Dose Level 2 will be achieved: 7.5 mg for 5-10 yrs, 15 mg for 11-17 yrs and 30 mg for > 18 yrs).<br><br>The patients will receive at T0 a first supply of study medication, and then additional supplies at each visit to cover the 24 weeks (i.e. 6-months) treatment period. Dose titration at Dose Level 2 should be completed by Day 15. If there are no tolerability issues, patients will continue on their maximum tolerated dose (MTD) though the final dose. In case of need, patients will receive a supply of study medication at the subsequent visit. The date, time, and ml of solution administered by the caregiver will be recorder in a patient's diary.<br><br>If intolerance develops at any time during the study, the Investigator may reduce patient's dose to a lower level, with a single dose interruption and administration every other day. Patient unable to tolerate treatment even after a dose interruption should be discontinued from the study. Discontinuation of the treatment must be also considered if: white blood cell count < 4000/$mm^3$ or an absolute neutrophil count < 2000/$mm^3$; hyponatremia (< 125 mmol/L); renal impairment (creatinine > 2 x ULN); hepatic impairment (AST, ALT, bilirubin > 2 x ULN); cardiovascular, respiratory, gastrointestinal, hematologic or other significant medical disorders (in addiction to other typical RTT); severe diabetes mellitus; QTcF interval on the ECG > 450 msec; severe hypotension.<br><br>These medications are not permitted before and/or during the trial: concurrent antidepressants or anxiolytic treatment with selective serotonin reuptake inhibitors (SSRIs); Monoamine Oxidase Inhibitors (MAOIs); anti-coagulant therapies (warfarin).<br><br>In accordance with ICH requirements, the Investigator must all times be able to account for all study drugs furnished to the institution. The Pharmacist/Investigator must sign the Drug Receipt Form, confirming that the IMP will be handled and stored properly. At the end of the study, it must be possible to reconcile delivery records with those of usage and returned stocks. |

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| | Account must be given of any discrepancy. Under no circumstances is the Investigator allowed to use the study medications other than those directed by the protocol. |
| **Study sites** | Patients will be recruited by four RTT reference Italian hospitals (Partners 1-4) which collectively care >420 RTT patients/year.<br>• **Coordinating centre** - Dipartimento Scienze della Vita, Università di Trieste. Trieste.<br>• **Partner 1.** Centro Epilessia – Unità Neurologia Pediatrica, ASST Ospedale Santi Carlo Paolo - Dipartimento Scienze della Salute, Università di Milano.<br>• **Partner 2.** Unità di Neuropsichiatria Infantile, IRCCS, Istituto Giannina Gaslini, Genova.<br>• **Partner 3.** Unità di Pediatria, Dipartimento della Donna e dei Bambini – Policlinico S. M. alle Scotte. Siena.<br>• **Partner 4**. Dipartimento di Patologia Umana dell'Adulto e dell'Età Evolutiva "Gaetano Barresi", Policlinico Universitario "G. Martino, University of Messina. Messina. |
| **Statistical Analysis Plan** | A Statistical Analysis Plan (SAP) will be finalized prior to database lock. The SAP will describe in detail study endpoints and statistical analyses, including the analysis of the primary as well as additional endpoints. In case changes to the original primary endpoint or of the original primary analyses occurs during the study, these changes will be the subject of a substantial protocol amendment.<br>All statistical analyses not prespecified and run after database lock will be considered additional/exploratory post-hoc analyses. |
| **Statistical Analysis** | The default significant level will be 0.05 (5%); confidence intervals will be 95% and all tests will be two-sided, |

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| | unless otherwise specified in the description of the analyses.<br><br>Summaries of continuous variables will include descriptive statistics (number of non-missing observations sample size [n], mean, standard deviation [SD], median, minimum and maximum) and for categorical variables (the number of non-missing observations frequency [n] and percentage), unless otherwise stated in the relevant section. Percentages will be based on the number of patients within the relevant analysis population, or the number of patients with data available where relevant.<br><br>The primary efficacy analysis will be performed for the ITT population.<br><br>The study hypothesis will be tested as following: change from baseline in Motor-Behavior Assessment Scale (MBAS) from prior and post mirtazapine therapy will be tested using paired t-test. The 2-sided p-value obtained from the paired t-test will be presented. Assumption of Normality will be investigated using Wilk-Shapiro test. If the p-value is greater than .05, it means we cannot reject the null hypothesis that a variable is normally distributed. If the data is found to be non-Normal, the paired t-test will be replaced by Wilcoxon signed rank test. The primary endpoint will be summarized descriptively using n, mean, median, SD, Q1, Q3, minimum, and maximum. Only those patients for whom we have MBAS assessment results available at both Baseline (Day 0) and End of Study (week 24) will be considered. If baseline (Day 0) or end of treatment (week 24) MBAS value is missing the patient is not included in the ITT population.<br><br>A repeated measure mixed model (MMRM) will be performed on the primary efficacy endpoint to test the factor/covariate effects for the ITT population. The MIXED model will include change from baseline in mean MBAS as dependent variable. A MIXED procedure, with an unstructured covariance matrix, will be used for the |

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| | analysis and the corresponding two-sided p-values for each of the covariates will be presented. If the fit of the unstructured covariance structure fails to converge, the following covariance structures will be tried in order until convergence is reached: Toeplitz with heterogeneity, compound symmetry, autoregressive with heterogeneity, Toeplitz, and autoregressive. Smaller value of AIC and BIC will be consider for selection of best fit for covariance structure.<br><br>The study will follow the Consolidated Standards of Reporting Trials (CONSORT) reporting guideline. The statistical analyses will be performed using SPSS version 29.0 (IBM SPSS Statistics for Windows, Version 29.0. Armonk, NY: IBM Corp). |
| **Trial Ethical considerations** | The study will start after the favourable opinion given by the Ethics Committee and approval from the regulatory authorities.<br><br>The study will be conducted in accordance with Good Clinical Practice (GCP) guidelines and in accordance with the ethical principles of the Declaration of Helsinki. |

## 2. Schedule of Activities (SoA)

| | **Screening** (up to 90 days before Day -1) | **T0** (Baseline day 0) | **I Phone follow-up contact** (2 weeks after T0) | **T1** (4 weeks ± 1 week from T0) | **II Phone follow-up contact** (8 weeks after T0) | **T2** (12 weeks ± 1 week from T0) | **III Phone follow-up contact** (16 weeks after T0) | **T3** (24 weeks ± 1 week from T0) | **T4 Follow-up** (1 month after the last dose) |
|---|---|---|---|---|---|---|---|---|---|
| Informed consent | X | | | | | | | | |
| Inclusion / Exclusion criteria | X | X | | | | | | | |
| Demography | X | | | | | | | | |
| Medical history (includes substance usage and Family history of premature CV disease) and current medical conditions | X | | | | | | | | |
| Vital signs (body temperature, pulse rate, respiratory rate, and blood pressure) | | X | | X | | X | | X | X |
| ECG (12-lead) | | X | | X | | X | | X | X |

| | **Screening** (up to 90 days before Day -1) | **T0** (Baseline day 0) | **I Phone follow-up contact** (2 weeks after T0) | **T1** (4 weeks ± 1 week from T0) | **II Phone follow-up contact** (8 weeks after T0) | **T2** (12 weeks ± 1 week from T0) | **III Phone follow-up contact** (16 weeks after T0) | **T3** (24 weeks ± 1 week from T0) | **T4 Follow-up** (1 month after the last dose) |
|---|---|---|---|---|---|---|---|---|---|
| Physical examination including anthropometric assessment | X | X | | X | | X | | X | X |
| Neurological examination | X | X | | X | | X | | X | X |
| Blood sample collection (Hematology, Biochemistry, and thyroid) | | X | | X | | X | | X | |
| Blood sample collection for Metabolism biomarkers | | X | | X | | X | | X | |
| Serum prolactin | | X | | X | | X | | X | |
| Serum ACTH and cortisol | | X | | X | | X | | X | |
| Serum pregnancy test (for post-pubertal patients) | X | X | | X | | X | | X | |
| Blood sample for neuronal plasticity (BDNF, GDNF PDGF) | | X | | | | X | | X | |

| | **Screening** (up to 90 days before Day -1) | **T0** (Baseline day 0) | **I Phone follow-up contact** (2 weeks after T0) | **T1** (4 weeks ± 1 week from T0) | **II Phone follow-up contact** (8 weeks after T0) | **T2** (12 weeks ± 1 week from T0) | **III Phone follow-up contact** (16 weeks after T0) | **T3** (24 weeks ± 1 week from T0) | **T4 Follow-up** (1 month after the last dose) |
|---|---|---|---|---|---|---|---|---|---|
| Polysomnographic exam (only in the centres of Genova and Milano) | | X | | | | | | X | |
| Actigraphy using a wrist sensor | | X | | X | | X | | X | |
| Biovital and cardiorespiratory monitoring at home (YouCare T-shirt, 24 h) | | X | | X | | X | | X | |
| Assessment of the severity (RCSS) | | X | | X | | X | | X | |
| Motor-Behavior Assessment Scale (MBAS) | | X | | X | | X | | X | |
| Mood assessment (ADAMS) | | X | | X | | X | | X | |
| Rett Syndrome Behavior Questionnaire (RSBQ) | | X | | X | | X | | X | |
| Purposeful Hand Function Scale (PHF) | | X | | X | | X | | X | |
| Sleep quality scale (SDSC) | | X | | X | | X | | X | |

| | **Screening** (up to 90 days before Day -1) | **T0** (Baseline day 0) | **I Phone follow-up contact** (2 weeks after T0) | **T1** (4 weeks ± 1 week from T0) | **II Phone follow-up contact** (8 weeks after T0) | **T2** (12 weeks ± 1 week from T0) | **III Phone follow-up contact** (16 weeks after T0) | **T3** (24 weeks ± 1 week from T0) | **T4 Follow-up** (1 month after the last dose) |
|---|---|---|---|---|---|---|---|---|---|
| Clinical Global Impression of Change (CGI-C) | | X | | X | | X | | X | X |
| Parenting Stress Index (PSI-SF) | | X | | | | X | | X | |
| Study treatment | | X | X | X | | X | | | |
| Study treatment compliance (patient's diary review) | | | X | X | X | X | X | X | |
| Return of study treatment kits and YouCare T-shirt | | | | | | | | X | |
| AE review | | X | X | X | X | X | X | X | X |
| SAE review | | X | X | X | X | X | X | X | X |
| Concomitant medications | | X | X | X | X | X | X | X | X |

Time Schedule

MONTH 1-6 Study set-up and Regulatory actions (AIFA, EC)

MONTH 7-9 Screening and Recruitment

MONTH 9-15 Treatment phase

MONTH 15-18 Follow-up phase and Data analysis

GANNT Chart of activities

| Phase | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Study set-up and Regulatory | x | x | x | x | x | x | | | | | | | | | | | | |
| Screening and Recruitment | | | | | | | X | x | x | | | | | | | | | |
| Treatment phase | | | | | | | | | x | x | x | x | X | x | x | | | |
| Follow-up phase and Data analysis | | | | | | | | | | | | | | | x | x | x | x |