Melatonin and pro-hypnotic effectiveness of the antidepressant Trazodone: a preliminary evaluation in insomniac mood-disorder patients

Gino Giannaccini[1(*)] ✉, Irene Masala[2*], Lionella Palego [2], Laura Betti[1], Bruno Pacciardi[2], Laura Palagini[2], Federica Luchini[2], Simone Belli[2], Antonio Lucacchini[1] and Mauro Mauri[2].

(*): These authors contributed equally to this work

[1]Department of Pharmacy, University of Pisa, Via Boannno 6, 56126 Pisa, Italy

[2]Department of Clinical and Experimental Medicine, University of Pisa, Via Savi 10, 56126 Pisa, Italy.

✉ **Corresponding author:**

Prof. Gino Giannaccini, Department of Pharmacy, University of Pisa, Via Bonanno 6, 56126-I, Pisa, Italy.

E-mail: gino.giannaccini@unipi.it

Authors' e-mail addresses:

Gino Giannaccini: gino.giannaccini@unipi;

Irene Masala: irene.masala@tiscali.it;

Lionella Palego: lionella.palego@unipi.it;

Laura Betti: laura.betti@unipi.it;

Bruno Pacciardi: bruno.pacciardi@gmail.com

Laura Palagini: lpalagini@tiscali.it

Federica Luchini: federica.luchini@alice.it

Simone Belli: bellis.md@gmail.com

Antonio Lucacchini: antonio.lucacchini@farm.unipi.it

Mauro Mauri: mauro.mauri@med.unipi.

Total manuscript words: 3978

Abstract

Objective: To preliminary investigate the link between the darkness hormone melatonin (MLT) and the pro-hypnotic effectiveness of the atypical antidepressant Trazodone (TRZ) in a group of mood disorder patients suffering of insomnia.

Design and Methods: The study's design comprised: i) the enrolment of insomniac outpatients, ii) baseline (t_0) psychiatric and biochemical examinations; iii) the subsequent patients' introduction into a treatment with TRZ for 3-4 weeks, followed by post-therapy re-evaluations (t_1). The MLT function was investigated by t_0/t_1 ELISA determinations of 6-hydroxy-MLT sulfate (6-OH-MLTs) levels in early-morning urines and HPLC analysis of morning MLT serum amount. Concomitantly, TRZ and its metabolite *m*-chloro-phenylpiperazine (*m*-CPP) were measured by HPLC in serum to monitor patients' compliance/metabolism.

Results: Seventeen insomniac outpatients, displaying mild symptoms of depression/anxiety resistant to antidepressants, completed TRZ therapy (dose:10-20 mg/day, bedtime). Serum TRZ levels (127± 57 ng ml^{-1}, mean ± SD) confirmed patients' compliance, while the anxiogenic metabolite *m*-CPP resulting almost undetectable. Moreover, the 6-OH-MLTs output was found increased at t_1 vs. baseline values (t_1: 58.4 ± 45.02 ng ml^{-1}; t_0: 28.6 ± 15.8 ng ml^{-1}; mean ±SD, $P < .05$) in 9 patients who recovered both insomnia and depression/anxiety ($P < .01$). Unresponsive subjects showed instead no post-therapy 6-OH-MLTs variation (t_1: 48.53 ± 50.70 ng ml^{-1}; t_0 : 49.80 ± 66.53 ng ml^{-1}). Morning MLT in serum slightly diminished at t_1 without reaching the statistical significance, not allowing therefore to define the patients' outcome.

Conclusions: This initial investigation encourages to explore MLT networks as possible correlates of TRZ pro-hypnotic responses.

Keywords: Insomnia, Mood disorders, Melatonin, Trazodone.

1.Introduction

The disruption of the sleep-arousal cycle negatively affects the individual quality of life, provoking nocturnal awakenings and/or excessive sleepiness during the day [1]. Since decades, sleep disturbances have been found tightly interlaced to neuropsychiatric disorders: more than 80% of depressed people experience at least one sleep symptom [2]. Among these, insomnia is particularly difficult to manage by psychiatrists, especially as concerns its diagnosis and suitable treatment. Blurred boundaries in fact exist between primary insomnia and that secondary to psychiatry diseases: if insomnia can be the core of the clinical symptoms of a mood-anxiety disorder, it can also be the almost unique prodromal symptom in an emerging psychiatric disease [3]; at the same time, a sleep dysfunction can be the residual sign in depressed patients after their remission, exposing them to a higher risk of relapse [4]. Sleep symptoms secondary to mood-anxiety diseases may be sub-threshold but invalidating in the long-term, while being often undiagnosed. Low-quality sleep and its persistence can significantly worsen the prognosis of patients with a major mood disorder [5], even increasing suicidality [6]. Another main aspect in the care of insomnia and depression is the possible arise of sleep side-effects during a therapy with antidepressants (ADs) owing to the psycho-stimulant effect of these compounds [7]. Therefore, such a varied portrayal suggests the need of adapted drugs/treatments. Beside GABAergic compounds, alternative pro-hypnotic drugs have been (re)discovered among the well-tolerated multifunctional ADs [8]. In particular, the "old" compound Trazodone (TRZ) [9,10], an atypical AD which behaves overall as a high-affinity antagonist of serotonin$_{2A/2C}$ (5-HT$_{2A/2C}$) receptors and as a lower affinity inhibitor of 5-HT reuptake (SARI), has shown a good pro-hypnotic efficacy in psychiatric patients. The action of TRZ on sleep is in fact attained at lower dosages than those provoking its antidepressant effect, presumably due to a combined anti-arousal effect resulting from its antagonism on both 5-HT$_{2A/2C}$ receptors [11] and α-adrenergic and/or H-histaminergic receptor subtypes [12]. Nevertheless, the precise mechanism of action which underlies this TRZ specific effect has never been fully elucidated yet. Interestingly, some authors have reported that the acute administration of TRZ, 50 mg at bedtime, raises the nighttime pineal melatonin (MLT) release in healthy volunteers [13]. This is an important remark, since the pineal gland function is attracting ever increasing attention in clinical psychiatry because of the recognized reciprocal influence of mood and sleep neurocircuitries [14-16]. The substantiation of a main role of MLT system in the sleep-promoting action of TRZ would in fact open up towards, on the one hand, a deeper knowledge of the physiopathology of insomnia and MLT release, while, on the other, towards the delineation of more targeted uses of this drug in the clinical practice.

The present work aimed therefore at preliminary assessing if TRZ effectiveness on sleep could be sustained by an action on MLT system in mood disorder patients suffering of insomnia. For this purpose, patients were enrolled and examined through suitable psychiatric rating scales before (baseline time, t_0) and after a therapy (post-therapy time, t_1) with TRZ, in concomitance to investigations on their melatonergic function. The MLT parameters measured in patients were: the amounts of 6-hydroxymelatonin sulfate (6-OH-MLTs) in early-morning urines, a metabolic index strongly correlated to nighttime pineal MLT secretion [17, 18], and morning MLT in serum [19]. The patients' compliance and TRZ metabolism were instead monitored through the determination of serum levels of TRZ and its anxiogenic metabolite *m*-chloro-phenylpiperazine (*m*-CPP) at t_1.

2. Experimental

2.1 Mood Disorder Patients

2.1.1 First recruitment interview

Mood disorder patients were enrolled at the Psychiatry Ambulatory Care of the Department of Clinical and Experimental Medicine of the University of Pisa, during their therapeutic follow-up. Recruitment occurred during the years 2012 and 2013 (winter and spring-time), within a total period of about 6 months, through an initial interview carried out accordingly to established inclusion/exclusion criteria. Inclusion criteria were: age ≥ 18 years; a diagnosis of major mood disorder obtained by the Structural Clinical Interview (SCID) for the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR) [20]; the presence of sleep disturbances at the time of the interview assessed by a "yes/no" test; the reading, endorsement and signature of the project previously approved by the Ethical Committee of the University of Pisa, in conformity to the World Medical Association Declaration of Helsinki. Exclusion criteria were: a normal sleep assessed by a "yes/no" test, co-morbidity with a neurological or neurodegenerative disease, the presence of renal and metabolic illnesses, alcohol/drug abuse, severe rheumatic/autoimmune disorders, pregnancy; a previous treatment with TRZ since at least 3 months.

2.1.2 Sleep and psychiatric rating scales

Each patient selected by the first interview underwent, within few days, programmed baseline (t_0) blood/urine tests and psychiatric examinations. The severity of symptoms was appraised by skilled psychiatrists working at the Psychiatry Unit of the University of Pisa through specific and validated rating scales: 1) the «Insomnia Severity Index» scale (ISI) [21]; 2) the 17-items «Hamilton Rating Scale for Depression» (HAMD-17) [22]; 2) the State-Trait Anxiety Inventory Form Y (STAI-Y1 and STAI-Y2) [23].

If deemed eligible on the basis of t_0 scores obtained by these 4 clinical evaluation scales and respective cut-off values [21-23], patients were definitely admitted to the survey. Thus, they were introduced into a period of 3-4 weeks under TRZ therapy and evaluated again by these same questionnaires at the end of the treatment (t_1) to complete the study. The treatment timing was established by considering the minimum time necessary to observe a possible patients' response on both sleep and mood-anxiety tonus. To avoid a seasonal influence on the study's results, t_0 and t_1 clinical/biochemical investigations were carried out on each patient by respecting photoperiod.

2.1.3 Drug treatment

Daily dosages of TRZ, taken at bedtime (10.00 - 11.30 pm), were set up in respect of patient's diagnosis, pharmacological treatment and clinical state examined at t_0. The established TRZ dosage was kept constant for the study's duration. Patients were allowed to maintain t_0 treatment with other psychoactive agents, provided that every change of drug and/or daily dosages was an exit criterion from the study. Patients taking benzodiazepines (BDZs) at doses not exceeding 1 mg/day and showing comparable clinical symptoms/scores to those who were not taking these compounds were also admitted to the investigation. The communication to the medical staff of arising illnesses or significant adverse effects during TRZ treatment also resulted in the exclusion from the study.

2.2 Biochemical investigations

2.2.1. Collection of biological samples

The day of the first interview, each patient had received an urine collection container and suitable guidelines to gather first-morning urines for the measurement of nighttime 6-OH-MLTs excretion. Briefly, patients were asked to collect first morning urines (07.00-08.30 am) at home when fasting, a procedure to be done the same day of the beginning of the study (t_0). Patients were also asked to undergo a t_0 peripheral venous blood withdraw of about 7 ml carried out between 08.15 and 09.30 am at the Psychiatry ward of the Department, before the clinical assessments. This same procedure was repeated at t_1. After urine and blood delivery, samples were immediately transported to the laboratory of the Department of Pharmacy, University of Pisa, in apposite bags for the transport of biological fluids. At the laboratory, urines were measured for clinical-chemistry parameters by means of an automated analyzer of reactive chemical strips (Aution-Micro, Menarini, Italy) and centrifuged at 2,000 g for 10 min at RT. Ensuing supernatants were taken and stored at -20°C until 6-OH-MLTs assay. Additionally, venous blood, collected into a serum-test tube provided at the Psychiatry ward, was left to clot and centrifuged at 2,600 g for 15-20 min, RT. Serum supernatants were then divided into aliquots directly processed for routine analyses and the measure of MLT, TRZ and *m*-CPP or stored at -80°C.

2.2.2 Urinary 6-OH-MLTs assay

The excretion of the sulfate hydroxyl metabolite of MLT in urines collected at t_0 and t_1 was evaluated by means of a competitive 96-wells plate solid-phase enzyme immunoassay (ELISA) kit (IBL International, GMBH, Germany). The day of the assay, urine samples were thawed, allowed to reach RT, diluted as indicated and directly added to plates for the determination of 6-OH-MLTs; after incuba-

tion/washing steps, samples were measured by a Wallac Victor2 multilabel/multitask 96-wells plate reader (PerkinElmer, USA) preset at 450 nm. Concentrations of urinary 6-OH-MLTs were then interpolated from the calibration curve equation and presented as ng ml^{-1} urine volume, standardized for urine concentration/dilution by the specific gravity correction [24].

2.2.3 Determination of MLT levels in serum

Morning circulating MLT was estimated in patients' serum extracted by dichloromethane, in accord to a previously described procedure [25]. The pineal hormone was then measured in all 17 patients at t_0 and t_1 by HPLC: the isocratic chromatography was achieved at a flow rate of 0.8 ml min^{-1} and RT, using an UltiMate™ 3000 HPLC system (Thermofisher Scientific,USA). The mobile phase was composed by 0.1M NaH_2PO_4, 4mM Sodium DecanSulfonate, 0.1mM Na_2-EDTA in 25% methanol (75:25, *v:v*), pH 5. All chemicals employed were of the best analytical HPLC grade (Romil, Cambridge, UK; Sigma-Aldrich, USA; Applichem, Germany).The stationary phase was a C18 Atlantis-T3 analytical column (3 µm beads, 4.5 x 150 mm), in-line with a C18 Atlantis T3 pre-column, both purchased from Waters Spa (USA). Injection volume was 100 µl using a Reodyne injection valve. Elution peaks were visualized through a double detection device, employing in-line electrochemical (Coulochem III, Esa, Thermofisher Scientific,USA) and fluorimetric (UltiMate™ FLD-3000, Thermofisher Scientific,USA) detectors. Electrochemical detection was carried out by means of a guard electrode (ECD_1) preset at +100 mV and a second, analytical palladium electrode (ECD_2) set at +550 mV. Fluorimetric analysis was performed at the λ_{ex} = 280 nm and the λ_{em} = 345 nm. Under present chromatographic conditions, the MLT standard (> 99%) eluted at a retention time of about 20-21 min, permitting to identify/quantify MLT in unknowns. The HPLC measure of serum MLT was validated using a highly sensitive ELISA kit (IBL International, GMBH, Germany) in 8 of the 17 subjects, both at t_0 and t_1. This kit is based upon a solid-phase immunoenzyme competitive principle employing biotin-labeled MLT, specific antisera, and streptavidin-conjugated phosphatase alkaline. Plates were read at 405 nm by the Wallac Victor2 spectrophotometer.

2.2.4 Trazodone and m-CPP assay in serum

Trazodone and its main metabolite *m*-CPP were quantified in t_1 sera by HP-LC. Trazodone and *m*-CPP were extracted by a liquid:liquid partition procedure under alkaline conditions, using *n*-hexane as the organic solvent and buspirone as the internal standard, following a slightly modified method from Patel et al [26]. After extraction, TRZ and *m*-CPP were analyzed by reversed-phase HP-LC and UV-photodiode (DAD) detection working at dual and variable wavelength [27].

2.3 Statistical analyses

Data are presented as the mean ± standard deviation (SD) and ranges (min and max values). Non-parametric inferential analyses were employed for both clinical and biochemical comparisons. Before (t_0)-and-after-(t_1) tests or unpaired data comparisons were carried out by the Wilcoxon and the Mann-Whitney tests, respectively; for correlations, the Spearman method was applied. The statistical threshold was always set at $P = 0.05$. For descriptive and inferential statistics, correlation/regression analyses or to calculate analytes' calibration curves/lines, the Graph-Pad Prism software was used (Graph-Pad Prism, version 5, San Diego, CA,USA).

Clinical scores appraised by the ISI, HAMD and STAI-Y questionnaires were normalized as the percent (%) reduction observed at t_1 vs. the t_0 value, considered the 100%; values greater at t_1 than t_0 were taken as a 0% reduction of symptoms.

3.Results

3.1 Clinical evaluations

Twenty-five outpatients were selected through the first interview. Non-eligibility at baseline by clinicians, follow-up discontinuity, inadequate information or protocol adherence, and therapy drop-out during treatment, resulted in the completion of the study until t_1 by 17 patients. Table 1 reports the baseline characteristics of these patients: overall, they were overweight middle-age women, presenting mild insomnia accompanied by mild-to-moderate depressive symptoms resistant to a therapy with ADs. Most of them had initial or central insomnia. Only two patients were insomniac in the last part of the night. Most patients also displayed acute and/or chronic anxiety symptoms defined by significantly higher scores for chronic/trait anxiety (STAI-Y2) (Table 2, Mann-Whitney test). Seven subjects presented also a clinical history of panic attacks. At the time of their recruitment, all patients were receiving ADs and/or other psychoactive agents since at least 2 months: i) non-bipolar depressed patients were taking one or two Selective-Serotonin Reuptake Inhibitors (SSRIs), except fluvoxamine, at doses comprised between 5 and 75 mg/day; ii) bipolar depressed patients were instead taking one SSRI, except fluvoxamine, (dose range: 5-150 mg/day) associated to a mood stabilizer among carbamazepine or lamotrigine (150-200 mg/day), lithium or valproate (450-900 mg/day) and/or one or two antipsychotic drugs among perfenazine and aripiprazole (4-8 mg/day) or quetiapine (300 mg/day). Seven of them were constantly taking BDZ within allowed dosage ranges.

The applied therapeutic protocol used a very low TRZ dosage, 10-20 mg/day, taken at bedtime, as previously indicated.

Table 2 reports results concerning comparisons between clinical scores obtained at t_1 vs. t_0 ones. The ISI, HAMD and trait-anxiety (chronic) STAI-Y2 scores were found significantly reduced at t_1 vs. t_0, while the state-anxiety (acute) STAI-Y1 scale only showed a tendency to decrease. Afterward, due to the purpose of this study, we divided patients into responders or non-responders, based on post-therapy ISI scores and ISI % reduction, considering the sleep response as hierarchical. Nine patients recovered insomnia very well: 8 lowered their ISI scores from sub-threshold/moderate ($8 \leq ISI \leq 21$) to clinically irrelevant ($ISI \leq 7$) at t_1 [21]; 1 patient showing an ISI reduction of about -40%, thus decreasing insomnia from moderate to sub-threshold [21], was also considered responder to TRZ treatment. Conversely, the remaining patients (n=8) were found un- or scarcely-responsive to low-dose TRZ displaying persistent sleep symptoms, in accord to ISI guidelines ($ISI_{t1} \geq 8$) [21]. Five of them even reported increased

t_1 ISI scores *vs.* t_0. This diverse sleep response was not conceivably due to a difference in TRZ dosages, since patients' daily doses did not significantly differ between subjects who responded to therapy (12.8 ± 4.4 mg) and those who did not (11.2 ± 2.3 mg) (Mann-Whitney, $P > 0.05$). The same result was found for TRZ dose normalized for body weight (given dose): 0.161 ± 0.067 mg kg^{-1} in responders vs. 0.122 ± 0.049 mg Kg^{-1} in non-responders ($P > 0.05$). As well, BDZ administration did not seem to affect TRZ effectiveness, since among the 8 non-responders 4 were taking BDZ during the study while the other 4 did not.

Figure 1(a) depicts ISI-score before-and-after comparisons separately conducted in sleep responders and non-responders. Figure 1(b-d) shows that sleep responders were also found to significantly improve HAM-D and STAI-Y2 ratings. From Figure 1, the % reduction of clinical scores in responders was: (a) ISI, 68 ± 24 % (36-100%); (b) HAMD-17, 51 ± 20% (33-91%); (c) STAI-Y1, 11 ± 8 % (0-20); (d) STAY-Y2, 16 ± 14 % (0-36%); in non-responders, it resulted instead: (a) ISI, 10 ± 13% (0-25%) ; (b) HAMD-17, 20 ± 24% (0-62.5%); (c) STAI-Y1, 11 ± 11 % (0-30%); (d) STAY-Y2, 8 ± 11% (0-29.5%). Furthermore, diagnosis and smoking behavior would exert an impact on clinical response: depression and non-smoking habit were prevalent in responsive subjects (n=7 depressed vs. n=2 bipolar subjects; n=7 non-smokers vs. n=2 smokers), while more smoker bipolar individuals were found among non-responders (n=5 bipolar vs. n=3 depressed subjects; n=6 smokers vs. n=2 non-smokers). Sleep responders and non-responders had similar t_0 scores, confirming the comparable insomnia/depression severity at baseline (data not shown, Mann-Whitney, $P > 0.05$).

3.2 Biochemical assessments

Routine analyses of t_0 and t_1 serum/urines reported standard check up clinical-chemistry parameters within the normal range, indicating that all patients were in good health at the time of the study. Urine specific gravity was always comprised between the Spotchem healthy range, 1.005 to 1.030, validating urine samples for further investigation.

Trazodone was detected in all 17 serum samples collected at t_1: the drug serum levels accounted for by 127 ± 57 ng ml^{-1}, demonstrating the good compliance to treatment. Under our experimental conditions, other drugs used in combination with TRZ did not interfere during HPLC analyses [27]. As shown in Figure 2(a), TRZ levels in ISI responders, 130 ± 66 ng ml^{-1}, were found comparable to those measured in non-responders, 116 ± 47 ng ml^{-1} ($P > 0.05$), accordingly to respective drug daily dosages. The main

metabolite of TRZ, the psycho-stimulant and anxiogenic compound *m*-CPP, was found almost undetectable in serum, as expected from the administered low dosages of the parent drug. The metabolite was detected in 3 subjects only where it accounted for by 22 ng ml^{-1}, on average. These 3 patients were all found responsive to the TRZ therapy. Figure 2(b) shows instead the positive trend of serum TRZ levels in respect to ISI % reduction (sleep response), when the analysis was performed in responder patients only.

When comparing urinary 6-OH-MLTs levels in the 17 patients altogether, these increased from t_0: 39.1± 46.6 ng ml^{-1}, to t_1: 53.6 ± 46.5 ng ml^{-1}, without reaching the statistical significance (Wilcoxon, $P > 0.05$). Figure 3(a,b) shows t_0/t_1 comparisons between 6-OH-MLTs levels separately carried out in responders and non-responders: metabolite excretion increased more than 60% on average at t_1 vs. t_0 in 8 of the 9 responders, resulting in a significant variation ($P < 0.05$), Figure 3(a). In contrast, t_0/t_1 urinary 6-OH-MLTs levels were found almost similar or decreased in patients unresponsive to TRZ, failing to reach the statistical threshold ($P > 0.05$), Figure 3(b).

As concerns MLT concentration in morning serum, this was reduced at t_1, albeit not significantly, in all patients: it measured 59.7 ± 43.2 pg ml^{-1} at t_0 vs. 51.8 ± 47.7 pg ml^{-1} at t_1, ($P > 0.05$). In Figure 3(c,d) are shown separate comparisons between serum MLT in responders vs. non-responders: in spite that the post- therapy values tended to be reduced in both groups, the statistical significance was not reached ($P > 0.05$).

All t_0 MLT-ergic parameters in responders were not different from non-responders (Mann-Whitney, $P > 0.05$). Diagnosis had no significant impact on MLT biochemistry, except a tendency towards lower serum or urine levels of MLT parameters in bipolar depressed patients (data not shown, Mann-Whitney, $P = 0.09$). On the whole, a high data dispersion was obtained by both ELISA and HP-LC techniques.

The comparison between serum levels of MLT obtained by HP-LC and those attained by ELISA, revealed a strong correspondence of these two methodologies: Spearman coefficient of correlations at t_0 and t_1 were, r=0.94 (P= 0.0006) and r=0.92 (P= 0.0012), respectively.

4.Discussion

To our knowledge, this pilot study addresses a topic which has not been investigated before, the involvement of MLT system in the low-dose efficacy of the atypical AD TRZ on insomnia in patients with mood disorders. At first, the study's design allowed to recruit patients displaying mild depression/anxiety symptoms resistant to AD therapies accompanied by insomnia. Then, we could observe that doses as low as 10-20 mg/day of TRZ were able to improve sleep and mood/anxiety symptoms in more than half of participants (≈ 53%). Since the antidepressant effect of TRZ is usually attained at dosages comprised between 150-500 mg/day [12], our results report a potential for TRZ at rebalancing/enhancing SSRIs' efficacy [7] through a synergic action on both sleep and mood. Other authors have instead reported a dissociation between insomnia and depression remission after TRZ administration at a much higher drug dosage, 100 mg/day [28]. Such divergences could be ascribed to the discrepant study's design, patients' selection or degree of symptoms' severity as well as to the different therapeutic protocols applied, without wash-out procedures in our work. Interestingly, responders had a prevalent diagnosis of atypical depression while, after the same therapy, a trend for bipolar patients amongst non-responders was observed. The good response to TRZ reported in patients with atypical depression might reflect the peculiar clinical features of this form such as the presence of symptoms overlapping seasonal-affective disorders [29]. Also smoking behavior seems to have an impact on clinical response, suggesting that bipolar and smoker individuals could represent a distinct cohort of patients. It is noteworthy to consider that the reduction of state-anxiety after the low-dose TRZ treatment was weaker than that reported for chronic, generalized anxiety, a finding which could also explain the incomplete remission of patients. Clinical improvement was supported by results obtained for TRZ serum levels, revealing the good compliance of all patients to the drug. Trazodone serum levels were, on average, quite proportional when compared to those reported in the current literature in patients taking higher dosages [30]. The main TRZ metabolite, *m*-CPP, was almost undetectable in our patients, resulting under the method's limit of quantification [27]. In fact, plasma/serum *m*-CPP is about the 1-10% of TRZ [31]. The 3 patients in whom serum *m*-CPP was quantified were also found to respond well to TRZ, suggesting that, at the maximal concentration determined herein, this metabolite cannot counteract the sedative effects of its parent compound [32]. Results on TRZ serum levels were congruent with a pharmacological action [12], concurrently discouraging the exclusive effect of placebo on patients' clinical responses and providing support to the reported biochemical results on MLT func-

tion. The main finding of this work consists in fact in the significantly increased excretion of the metabolite 6-OH-MLTs measured in first morning urine of responder patients only, reflecting an enhanced nighttime production of the pineal hormone in these subjects and suggesting an interaction between TRZ and MLT system, as previously observed by Morera and co-authors [13]. Our basal urinary 6-OH-MLTs levels were, on average, about 1.5-2 folds lower than those measured in healthy Italian volunteers [33] as well as in non depressed controls from other countries [18,34]. Conversely, morning levels of MLT in serum reported in our study were 1.5-2 folds higher, on average, than those measured in healthy subjects by others: using the ELISA method, Keskin et al. [35] showed a mean circulating morning MLT values of about 30 pg ml^{-1} in control, non-depressed subjects. Khaleghipour et al. [36] showed much lower MLT serum levels at 8.00 a.m. in healthy subjects (about 10 pg ml^{-1}), still accompanied by a 2-fold higher MLT in non-medicated depressed patients. Beside, Khaleghipour and coauthors reported lower nighttime MLT serum levels in subjects with depression [36]. Thus, theirs and our results would imply a blunted MLT release during the night with elevated MLT levels in the morning, probably due to a delayed nighttime peak of the pineal hormone. A similar pattern of MLT release has been already found in depressed subjects, even if not unanimously replicated [37]. Morning MLT showed only a tendency to reduction after TRZ therapy in our patients, probably due to a prevailing action of the drug on nighttime MLT release [13]. The negative result obtained for morning MLT serum levels could also derive from the inherent limitation of a single blood sample in the day light-phase, at low pineal activity, in removing interferences linked to the well-known high inter-individual variability of the MLT circadian metabolic profile, influenced by genetics, life-style and environmental factors, in addition to the concomitant treatment with SSRIs or other drugs [38,39]. Indeed, as early-morning 6-OH-MLTs reflects the sum of nighttime pineal secretion, this parameter would be a more robust indicator of the drug's action, at its low doses, on MLT system. A main issue as concerns urinary 6-OH-MLTs for appraising pineal function resides in the fact that this represents an indirect and cumulative measure of MLT production, being the product of II pass sulfoconjugation enzymes; by contrast, for logistic and ethical problems, urine collection is surely a non-invasive and inexpensive procedure [15].

5. Conclusions

To sum up, our pilot examination, considering its intrinsic limitations, would support the role of circadian MLT as a biological indicator of pro-hypnotic/antidepressant benefits of TRZ and encourages to pursue the study. If confirmed, present findings would contribute to better define the molecular effectors of MLT on sleep and mood tonus [40], being also helpful to pharmacologists and clinicians.

Acknowledgements

This pilot study is part of a spontaneous investigation on the biochemical substrates which could underlie the clinical response to atypical antidepressant drugs in patients with mood and sleep disorders.

Authors' contribution

GG, IM and MM conceived the study design and coordinated all investigation steps, including the manuscript's supervision/preparation and ethical aspects. GG, LPo and LB organized and developed the study; BP, LPi, FL and SB performed all the psychiatric and sleep interviews/evaluations; LPo and LB collected biological samples and conducted laboratory experiments; LPo, LB and IM wrote the manuscript; AL provided relevant contribution to define experimental procedures and to prepare this paper. All authors read and approved the final manuscript.

Conflict of Interests

The authors declare no conflict of interest.

References

[1] J.L. Hossain, C.M. Shapiro, The prevalence, cost implications, and management of sleep disorders: an overview, Sleep Breath 6 (2002) 85-102.

[2] J.D. Nutt , S. Wilson, L. Paterson, Sleep disorders as core symptoms of depression, Dialog Clin Neurosci 10 (2008) 329-336.

[3] T. Paunio, T. Korhonen, C. Hublin, et al., Poor sleep predicts symptoms of depression and disability retirement due to depression, J Affect Disord 172 (2015) 381–389.

[4] A.A. Nierenberg, M.M. Husain, M.H. Trivedi, et al., Residual symptoms after remission of major depressive disorder with citalopram and risk of relapse: a STAR*D report, Psychol Med 40 (2010) 41-50.

[5] F. Vellante, M. Cornelio, T. Acciavatti, et al., Treatment of resistant insomnia and major depression, Clin Ther 164 (2013) 429-435.

[6] R.A. Bernert, T.E. Joiner, Sleep disturbances and suicide risk: A review of the literature, Neuropsychiatr Dis Treat 3 (2007) 735–743.

[7] C.M. Dording, D. Mischoulon, T.J. Petersen, et al., The pharmacologic management of SSRI-induced side effects: a survey of psychiatrists, Ann Clin Psychiatry 3 (2002) 143-147.

[8] S. Stahl, Multifunctional drugs: a novel concept for psychopharmacology, CNS Spectr 14 (2009) 71-73.

[9] T.L. Schwartz, V. Goradia, Managing insomnia: an overview of insomnia and pharmacologic treatment strategies in use and on the horizon, Drugs Context (2013) 212257.

[10] A. Fagiolini, A. Comandini, M. Catena Dell'Osso, et al., Rediscovering Trazodone for the Treatment of Major Depressive Disorder, CNS Drugs 26 (2012) 1033–1049.

[11] J.M. Monti, Serotonin control of sleep-wake behavior, Sleep Med Rev.15 (2011) 269-281.

[12] S. Stahl, Mechanism of action of Trazodone: a multifunctional drug, CNS Spectr 14 (2009) 536-546.

[13] A.L. Morera, P. Abreu-Gonzalez, M. Henry, et al., Trazodone hypnotic effect and nocturnal melatonin secretion, J Clin Psychopharmacol 29 (2009) 97-99.

[14]M.A. Quera Salva, S. Hartley, F. Barbot, et al., Circadian Rhythms, Melatonin and Depression, Curr Pharmaceut Design 17 (2011) 1459-1470.

[15] L. Carvalho, C. Gorenstein, R. Moreno, et al., Effect of antidepressants on melatonin metabolite in depressed patients, J Psychopharm 23 (2009) 315-321.

[16] V. Srinivasan, Z. Rahimah, O. Zahiruddin, et al., Melatonergic Drugs for Therapeutic Use in Insomnia and Sleep Disturbances of Mood Disorders, CNS & Neurol Disord - Drug Targets 11 (2012) 180-189.

[17] J. Arendt, C. Bojkowski, C. Franey, et al., Immunoassay of 6-hydroxymelatonin sulfate in human plasma and urine: abolition of the urinary 24-hour rhythm with atenolol, J Clin Endocrinol Metab 60 (1985) 1166-1173.

[18] M.R. Cook, C. Graham, R. Kavet, et al., Morning urinary assessment of nocturnal melatonin secretion in older women, J Pineal Res 28 (2000) 41-47.

[19] M. Crasson, S. Kjiri, A. Colin, K. Kjiri, et al., Serum melatonin and urinary 6-sulfatoxymelatonin in major depression, Psychoneuroendocrinology 29 (2004) 1–12.

[20] M.B. First, R.L. Spitzer, M. Gibbon, et al., Structured Clinical Interview for DSM-IV-TR Axis I Disorders, Research Version, Patient Edition (SCID-I/P). New York: Biometrics Research, New York State Psychiatric Institute (2002).

[21] C.H. Bastien, A. Vallières, C.M. Morin, Validation of the Insomnia Severity Index as an outcome measure for insomnia research, Sleep Med 2 (2001) 297-307.

[22] M. Zimmerman, J.H. Martinez, D. Young, et al., Severity classification on the Hamilton Depression Rating Scale, J Affect Disord 150 (2013) 384-388.

[23] L.J. Julian, Measures of anxiety, Arthritis Care Res 63 (2014) S467-S472.

[24] E.J. Cone, Y.H. Caplan, F. Moser, et al., Normalization of Urinary Drug Concentrations with Specific Gravity and Creatinine, J Anal Toxicol 33 (2009) 1-7.

[25] V. Rizzo, C. Porta, M. Moroni, et al., Determination of free and total (free plus protein-bound) melatonin in plasma and cerebrospinal fluid by high-performance liquid chromatography with fluorescence detection, J Chromatogr B Analyt Technol Biomed Life Sci 774 (2002) 17-24.

[26] B.N. Patel, N. Sharma, M. Sanyal, et al., High throughput and sensitive determination of trazodone and its primary metabolite, m-chlorophenylpiperazine, in human plasma by liquid chromatography-tandem mass spectrometry, J Chromatogr B Analyt Technol Biomed Life Sci 871 (2008) 44-54.

[27] L. Palego, G. Giannaccini, I. Masala, et al., Analysis of Trazodone and *m*-CPP in human serum by RPLC and UV-photodiode array detection, J Appl Basic Res Int 12 (2016) 85-95.

[28] H. Kaynak, D. Kaynak, E. Gozukirmizi, et al., The effects of trazodone on sleep in patients treated with stimulant antidepressants, Sleep Med 5 (2004) 15-20.

[29] M.F. Juruena, A.J. Cleare, Overlap between atypical depression, seasonal affective disorder and chronic fatigue syndrome, Rev Bras Psiquiatr 29 (2007) S19-26.

[30] L. Mercolini, C. Colliva, M. Amore, et al., HPLC analysis of the antidepressant trazodone and its main metabolite m-CPP in human plasma, J Pharm Biomed Anal 47 (2008) 882-887.

[31] C. Hiemke, P. Baumann, N. Bergemann, et al., AGNP Consensus Guidelines for Therapeutic Drug Monitoring in Psychiatry: Update 2011, Pharmacopsychiatry 44 (2011) 195-235.

[32] R. Rajkumar, D.K. Pandey, R. Mahesh, et al., 1-(*m*-Chlorophenyl)piperazine induces depressogenic-like behaviour in rodents by stimulating the neuronal 5-HT(2A) receptors: proposal of a modified rodent antidepressant assay, Eur J Pharmacol 608 (2009) 32–41.

[33] P. Cocco, M.E. Cocco, L. Paghi, et al., Urinary 6-sulfatoxymelatonin excretion in humans during domestic exposure to 50 hertz electromagnetic fields, Neuroendocrin Lett 26 (2005) 1-7.

[34] R. Mahlberg, A. Tilman, L. Salewski, et al., Normative data of the daily profile of urinary 6-sulfatoxymelatonin in healthy subjects between the ages of 20 and 84, Psychoneuroendocrinology 31 (2006) 634-641.

[35] G. Keskin, D. Keskin, A. Inal, et al., Serum Melatonin levels in Sjögren patients, J Immunol 192 (2014) S 184.19

[36] S. Khaleghipour, M. Masjedi, H. Ahade, et al., Morning and nocturnal serum melatonin rhythm levels in patients with major depressive disorder: an analytical cross-sectional study, Sao Paulo Med J 130 (2012) 167-172.

[37] V. Srinivasan, M. Smits, W. Spence, et al., Melatonin in mood disorders, World J Biol Psychiatry 7: (2006) 138-151.

[38] L.M. Nogueira, J.N. Sampson, L.W. Chu, et al., Individual variations in serum melatonin levels through time: implications for epidemiologic studies, PLoS One 8 (12) (2013) e83208.

[39] K. Brøsen, Some aspects of genetic polymorphism in the biotransformation of antidepressants, Therapie 59 (2004) 5-12.

[40] V. Simonneaux, C. Ribelayga, Generation of the melatonin endocrine message in mammals: a review of the complex regulation of melatonin synthesis by norepinephrine, peptides, and other pineal transmitters, Pharmacol Rev 55 (2003) 325-395.

Figure legends

Figure 1 – Scores on psychiatric rating scales in sleep responders and non-responders. Responders showed: (a) ISI, t_0 = 12.2 ± 2.6; t_1= 3.45 ± 2.9; (b) HAMD-17, t_0 = 17.4 ± 5.1; t_1: 8.8 ± 4.7; (c) STAI-Y1, t_0: 45.11 ± 12.45; t_1: 40.56 ± 10.44; (d) STAI-Y2, t_0 = 58.0 ± 9.0; t_1: 48.0 ± 10.0. Non-responders displayed: (a) ISI, t_0: 13.0 ± 4.0; t_1: 13.0 ± 3.6; (b) HAMD-17, t_0: 17.0 ± 2.0; t_1: 13.2 ± 5.0; (c) STAI-Y1, t_0: 44.5 ± 10.0; t_1: 42.0 ± 6.1; (d) STAI-Y2, t_0: 48.0 ± 8.1; t_1: 46.0 ± 5.1. Wilcoxon, (**), P< 0.01; (*), P < .05; (°) nearly significant, P< 0.10; (ns), P > 0.05.

Figure 2- Serum levels of Trazodone. (a) TRZ levels in responders and non-responders, separately; (b) Spearman correlation between TRZ levels and ISI % reduction in responders. Mann-Whitney, (ns), not significant, P > 0.05; Spearman (*), P < 0.05. The line represents the trend of the relationship between variables, from the best fit of linear regression analysis of data.

Figure 3- MLT parameters in responder and non-responder patients. Urinary 6-OH-MLTs in responders increased from, (a): t_0, 29.60 ± 15.83 to t_1, 58.04 ± 45.02 ng ml^{-1}; in non-responders it measured (b): t_0, 49.8 ± 66.53 vs. t_1 48.53± 50.68 ng ml^{-1}. Serum MLT in responders was, (c): t_0, 59.8 ± 42.5 vs. t_1, 52.3 ± 38.9 pg ml^{-1}; in non-responders, (d): t_0, 59.6 ± 46.9 vs. t_1, 51.2 ± 58.8 pg ml^{-1}. Wilcoxon, (*), P < 0.05; (ns), not significant, P > 0.05.