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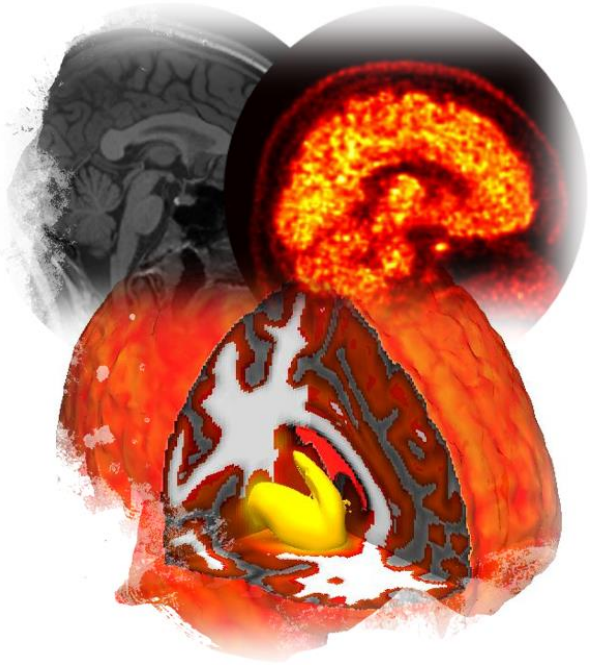

PhD Thesis

The serotonin 4 receptor binding as a novel imaging marker in major depressive disorder and the association to antidepressant treatment response

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Dansk resumé

I dag baseres behandlingen af depression typisk på en klinisk vurdering, og der mangler således relevante biologiske markører for at vejlede valget af antidepressiv behandling. Konventionel farmakologisk behandling retter sig primært mod monoamin-systemet, hvor Selective Serotonin Reuptake Inhibitors (SSRI) der påvirker serotonin-systemet, er førstevalg. Mens nogle patienter har gavn af medicinen, oplever omkring 30-50% ikke tilstrækkelig respons efter tre måneders behandlingsforsøg med et SSRI, og det er ikke muligt at forudsige hvem der ikke responderer. Depression er sandsynligvis en biologisk heterogen sygdom, hvilket kan være med til at forklare det varierende behandlingsrespons. En bedre viden om de biologiske mekanismer der ligger til grund for depression, og en forklaring på det varierende behandlingsrespons, er blevet efterlyst i flere årtier.

[^{11}C]SB207145 er en Positron Emission Tomography (PET) radioligand som binder til serotonin 4 receptoren (5-HT$_4$R) i hjernen. Både prækliniske og kliniske studier har vist, at densiteten af 5-HT$_4$R er omvendt relateret til serotonin-niveauerne i hjernen. Denne relation giver os en unik mulighed at måle både 5-HT$_4$R og serotonin-niveauerne i hjernen (udtrykt som 5-HT$_4$R binding) hos patienter med depression *in vivo*.

Formålet med dette PhD-studie er at:
1. Undersøge forskelle i 5-HT$_4$R PET binding hos patienter med depression sammenlignet med raske kontroller.
2. Undersøge om 5-HT$_4$R PET binding ved baseline kan forudsige behandlingsrespons efter 8 ugers antidepressiv behandling.
3. Undersøge ændringer i 5-HT$_4$R PET binding efter 8 ugers antidepressiv behandling.

Et hundrede ubehandlede patienter med moderat til svær depression samt 91 matchede raske kontroller blev inkluderet i et åbent, ikke-randomiseret, klinisk studie. 5-HT$_4$R non-displaceable binding potential (BP_{ND}) blev målt med PET [^{11}C]SB207145 ved baseline. Alle patienter startede efterfølgende behandling med escitalopram (SSRI) og blev fulgt med kliniske samtaler ved uge 1, 2, 4, 8 og 12. Fyrre patienter blev re-skannet efter 8 ugers antidepressiv behandling. Behandlingsresponset blev målt med Hamilton Depression Rating Scale 6 items ($HAMD_6$).

Vi fandt at patienter med depression havde 7-8% lavere 5-HT_4R BP_{ND} sammenlignet med raske kontroller i neocortex, hippocampus, nucleus caudatus og putamen. Vi fandt også, at patienter der responderede godt på behandlingen (remitters) havde 8-10% lavere binding end raske kontroller ved baseline, mens patienter der ikke responderede på behandlingen (non-responder) ikke havde forskelligt bindingsniveau ved baseline sammenlignet med raske kontroller. Baseline 5-HT_4R bindingen var ikke en god prædiktor for behandlingsrespons ved uge 8. Efter 8 ugers behandling var bindingen i neostriatum faldet 9% uafhængig af det kliniske behandlingsrespons.

Baseret på det inverse forhold mellem 5-HT_4R binding og serotonin-niveauerne i hjernen kunne vores resultater tyde på, at patienter som remitterer efter SSRI behandling har et højere serotoninniveau allerede før behandling; måske som udtryk for en kompensatorisk mekanisme mod forstyrrelser i serotoninsystemet i hjernen. En alternativ forklaring er en direkte effekt af lavere kapacitet for 5-HT_4R agonisme hos disse patienter. Lavt 5-HT_4R niveau kunne således være udtryk for en trait eller state markør for depression i en gruppe af patienter, som kunne udgøre en ”serotonerg” subtype af depression. Non-respondere med normal 5-HT_4R binding kunne i stedet have en ”ikke-serotonerg” depression, hvor den underliggende patologi ikke er koblet til en forstyrrelse af serotonin-signaleringen i hjernen.

Vores billeddiagnostiske fund af mulige biologiske subtyper indenfor depression kan på sigt hjælpe både med stratificering af depression som sygdom samt udviklingen af mere målrettet antidepressiv medicinsk behandling.

Thesis summary

Treatment choices in major depressive disorder (MDD) are mostly based on clinical evaluations, and there are no relevant biomarkers to guide treatment selection. Conventional pharmacotherapy is primarily targeting the monoaminergic system, especially the serotonin system using Selective Serotonin Reuptake Inhibitors (SSRIs) as first line treatment. Even though some patients benefit from SSRI treatment, for unknown reasons, 30-50% does not respond sufficiently to serotonergic acting drugs and efforts to predict who these patients are have generally failed. MDD is regarded to be a heterogenous disorder, which might influence the various treatment response. Better knowledge of de underlying biological mechanisms in MDD and its possible impact on treatment response has been a research priority for decades. [^{11}C]SB207145 is a Positron Emission Tomography (PET) radioligand that binds to the serotonin 4 receptor (5-HT$_4$R) in the brain. Preclinical and clinical studies have shown that the density of the 5-HT$_4$R binding is inversely related to the serotonin levels in the brain *in vivo*. This opens for a unique possibility to study the association between 5-HT$_4$R levels in the brain and disorders with a serotonergic involvement, such as MDD.

The aim of this PhD study was to:

1. Study differences in 5-HT$_4$R PET binding in patients with MDD compared with healthy controls.
2. Predict treatment response in MDD after 8 weeks of serotonergic treatment, based on baseline 5-HT$_4$R PET binding.
3. Study changes in 5-HT$_4$R PET binding after 8 weeks of serotonergic antidepressant treatment.

We included 100 antidepressant-free patients with moderate to severe MDD and 91 matched healthy controls. The 5-HT$_4$R non-displaceable binding potential was assessed with PET [^{11}C]SB207145. Forty patients were rescanned after 8 weeks of antidepressant treatment. All patients started treatment with escitalopram and received clinical follow-up visits after 1, 2, 4, 8 and 12 weeks. Treatment response was monitored with Hamilton Depression Rating Scale 6 items (HAMD$_6$). The primary response groups were categorized as remitters or non-responders at week 8.

We found that patients had 7-8% lower 5-HT_4R binding at baseline when compared with healthy controls. After stratification according to response status at week 8, we found that non-responders did not differ in 5-HT_4R baseline binding compared with healthy controls, whereas patients who obtained remission had 8-10% lower 5-HT_4R baseline binding compared with healthy controls. The binding at baseline was not suited to predict treatment response after 8 weeks treatment. The rescan results showed that the 5-HT_4R binding was reduced with 9% in neostriatum for all rescanned patients, regardless of the clinical treatment outcome.

Our data suggests that depressed patients who remit to SSRI treatment have higher serotonin levels before treatment compared to healthy controls, perhaps as an indicator of a disturbance in the serotonin system. Alternatively, or in addition, these patients could be characterized by low capacity for 5-HT_4R agonism. We propose that low 5-HT_4R binding could be a trait or a state marker for depressed patients who remit to SSRI, and that these might comprise a "serotonergic-related" subtype of MDD. Non-responders with normal 5-HT_4R binding might instead have a "non-serotonergic"-related depression, perhaps because the underlying pathophysiology is distinct from alterations in the central serotonergic system.

The identification of possible biological subtypes in MDD based on 5-HT_4R molecular neuroimaging might help in stratification of patients with MDD as well as aid in the development of future antidepressant precision medicine.

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- Kristin, Copenhagen, Denmark. April 2020.

List of manuscripts

Manuscript I:

Kristin Köhler-Forsberg, Anders Jørgensen, Vibeke H. Dam, Dea S Stenbæk, Patrick M. Fisher, Cheng-Teng Ip, Melanie Ganz, Henrik E Poulsen, Annamaria Giraldi, Brice Ozenne, Martin B Jørgensen, Gitte M Knudsen, Vibe G Frokjaer. Predicting treatment outcome in Major Depressive Disorder using serotonin 4 receptor PET brain imaging, functional MRI, cognitive-, EEG-based and peripheral biomarkers: a NeuroPharm open label clinical trial protocol. *Frontiers in Psychiatry*. Under review

Manuscript II:

Kristin Köhler-Forsberg, Brice Ozenne, Elizabeth Landman, Søren V. Larsen, Asbjørn S. Poulsen, Vibeke H. Dam, Cheng-Teng Ip, Anders Jørgensen, Michal Meyer, Hans R. L. Eiberg, Claus Svarer, Martin B. Jørgensen, Vibe G. Frokjaer *, Gitte M. Knudsen *. Evidence for a serotonergic subtype of major depressive disorder.

Abbreviations

ΔBP_{ND}: Change in Non-displaceable Binding Potential

5-HT: Serotonin

5-HT_4R: Serotonin 4 receptor

5-HTTLPR: Serotonin Transporter-linked Promotor Region

AUC: Area Under the Curve

B_{avail}: Concentration (density) of available receptors, *in vivo*

BMI: Body Mass Index (kg/m^2)

BP: Binding Potential

BP_{ND}: Non-displaceable Binding Potential

cAMP: cyclic adenosine monophosphate

CI: Confidence Interval

DSM-V: Diagnostic Statistics Manual, version 5

f_{ND}: fraction of free radioligand in the non-displaceable compartment

GCP: Good Clinical Practice

$HAMD_{17}$: Hamilton Depression Rating Scale 17 items

$HAMD_6$: Hamilton Depression Rating Scale 6 items

ICD-10: International Statistical Classification of Diseases and Related Health Problems, version 10

K_D: Equilibrium dissociation constant

LVM: Latent Variable Model

MDD: Major Depressive Disorder

MDI: Major Depression Inventory

MRI: Magnetic Resonance Imaging

p.adj: Adjusted p-vaule

PET: Positron Emission Tomography

$r\Delta HAMD_{6:}$ Relative change in delta Hamilton Depression Rating Scale 6 items

ROC curve: Receiver Operating Characteristic curve

SD: Standard Deviation

SERT: Serotonin transporter

SNRI: Serotonin Norepinephrine Reuptake Inhibitor

SRTM: Simplified Reference Tissue Model

SSRI: Selective Serotonin Reuptake Inhibitor

STAR*D: Sequenced Treatment Alternatives to Relieve Depression

Introduction

Major Depressive Disorder – epidemiology

Major Depressive Disorder (MDD) is one of the most frequent psychiatric disorders, affecting around 163 million people worldwide (264 million including dysthymia). It is also one of the leading causes for years lived with disability according to the global burden of disease study of 2017 [1]. The large international World Mental Health survey including 18 countries (n=89.037) found an average 12-months prevalence of around 6%, and the average age of onset was 25.7 in high- and 24.0 in low/middle-income countries [2]. Lifetime prevalence of MDD was reported to 10.8% in a recent large meta-analysis including more than 1 million people across 30 countries [3], but higher estimates of 17.1 % has also been reported [4], although these figures might be misleading due to methodological issues such as recall-bias and underestimation. For comparison, a Danish population-based register study found that life-time risk for receiving treatment for MDD (single or recurrent) was 9 % for men and as much as 15.5% for women. Evidently, MDD is a disorder that affects both sexes at all ages including young adults, supposedly at a critical time in life for decisions regarding future directives (e.g., becoming independent, choosing education, starting families) which may be highly influenced in a negative way. MDD is also associated with increased risk of somatic conditions like stroke, cardiovascular, metabolic diseases and cancer [5]. A recent register-based study of the Danish population found that mood disorders including MDD was associated with a decrease in life-expectancy with 6 and 8 years for women and men, respectively [6]. At its worst, MDD can result in death by suicide, which is the second leading cause of mortality for young adults in the United States [7]. Altogether, MDD is a common, wide-spread, burdensome and potentially life-threatening disorder.

Diagnostic criteria for MDD

There are two recognized major diagnostic tools used to diagnose MDD: 1) the International Statistical Classification of Diseases and Related Health Problems version 10 (ICD-10) issued from the WHO and 2) the Diagnostic and Statistical Manual of Mental Disorders version 5 (DSM-V) published by the American Psychiatric Association. At its core, MDD is characterized by sustained depressive symptoms for at least two weeks, including depressed mood, fatigue/loss

of energy and loss of interest in activities that are normally found to be pleasurable. Along with this, excessive feelings of worthlessness and guilt, changes in appetite and weight, sleeping alterations (hyper- or insomnia), cognitive disturbances and recurrent suicidal ideations may occur in mixed constellations. Different clinical subtypes of MDD have been suggested, such as the melancholic and atypical type to mention a few [5]. The two sets of diagnostic criteria are much alike, but they also reflect the issue of heterogeneity in MDD, since more than 227 different combinations of depressive symptoms (combinations with ≥5 symptoms in DSM-IV) still describe the same disorder [8]. It has been widely argued that MDD is not a unidimensional disorder with one underlying cause but rather a complex interplay of biological, psychosocial, behavioral and cultural factors [9,10]. Even after intense research and various suggestions of disease pathways, a fundamental understanding of the etiology and pathophysiology in MDD remains largely unknown [11].

The monoamine hypothesis of MDD - and its critics

Serotonin (5-hydroxytryptamine, 5-HT) is a monoaminergic neurotransmitter with central and peripheral actions, thought to play a key role in MDD. Out of serendipity in the late 1950s, it was found that drugs such as Imipramine (a derivative of antihistamines) and Iproniazid (a monoamine oxidase inhibitor used against tuberculosis) also exerted antidepressant effects [12,13]. From the research on their action on monoaminergic receptors rose the monoamine hypothesis which postulates causality between depleted levels of brain monoamine neurotransmitters and MDD [14,15]. Consequently, restoration of such a "chemical imbalance" would act therapeutically. Historically, the development of medications targeting especially the 5-HT system, e.g. selective serotonin transporter inhibitors (SSRIs), have been widely used and still hold a leading position among antidepressant drugs. The neuropharmacological action of SSRIs is to block the 5-HT transporter (SERT) and thereby increase extra-cellular 5-HT availability in the synaptic cleft [16], thus counteracting a presumed (according to the monoamine hypothesis) 5-HT deficiency. Further support for a direct involvement of 5-HT in MDD has come from studies of monoamine depletion paradigms, which show worsening of depressive symptoms in previously depressed patients [17]. On the other hand, it has not been possible to provoke a depressive episode in healthy volunteers, nor worsen depressive symptoms in already depressed individuals through monoamine depletion [18,19]. The monoamine hypothesis does also not provide a full explanation for how e.g., SSRIs yield antidepressant effects, since they acutely elevate extra synaptic 5-HT

levels (e.g. found in animal studies using microdialysis [20]) but the clinical antidepressant effect can be delayed with several weeks [21,22].

Obstacles in MDD treatment strategies

Antidepressant treatment choices are mainly based on a clinical evaluation of the patient. A crucial problem is the lack of predictability of whether a given patient will benefit from a specific antidepressant drug. In clinical practice, trial-and-error drug management directs treatment strategies in lack of significant and relevant biological markers to support a pharmacological selection. At an individual level, the delay from initiating antidepressant pharmacotherapy to actual effective response might increase the risk for premature discontinuation of the treatment, especially if side effects prevail or if the patient needs to switch drug class [23]. Even though SSRIs are the first line treatment to MDD, only about one third of patients achieve remission after a first 12 week trial with a common SSRI, as found in the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study Level 1, the largest out-patient clinical trial for treatment response in MDD to date [24]. After four trials of various antidepressant treatment approaches (including cognitive therapy and drug augmentations) a staggering 1/3 of patients were still not in remission. Efforts have been made to identify subtypes based on clusters of symptoms that may have distinct etiological pathways, and even though some studies have identified patients that are more or less likely to respond to certain drugs, the ability to predict treatment response has generally been unsuccessful [10]. Overall, there is a need to adapt a more personalized treatment approach instead of a "one-size-fits-all" model, including stratification of MDD into subtypes that e.g. could be based on quantitative biological measures through molecular brain imaging.

Positron emission tomography and kinetic modeling

The use of neuroimaging technologies such as positron emission tomography (PET) has vastly increased our knowledge of psychiatric disorders and contributed to the field of clinical neuroscience [25]. PET has introduced an invaluable and unique method for studying neurobiological conditions *in vivo*, for example neuroreceptor distribution by quantification of PET radioligands that specifically bind to a target of interest. A PET radioligand is a molecule tagged with a positron-emitting isotope (e.g. ^{11}C, ^{18}F, ^{15}O and ^{13}N). There are a few requirements

for a radioligand; the injected amount must be at tracer levels and hence not induce a physiological or pharmacological response; the ligand is in steady-state (or freely diffusible) with the endogenous target; and there is no isotope effect (no changes in properties of the radioligand due to adding the nuclide) [26]. The radioligand is injected into the bloodstream and distributes to the body and brain while the isotope decays at a fixed rate (e.g., half-life for ^{11}C is ~20 min). The unstable isotope then randomly emits a positron, which travels a short distance in the tissue and then annihilates with an electron, resulting in two emitted gamma-rays (photons) in opposite (180º) directions, each with an energy level of 511 keV. The photons can then be detected by separate PET-detector crystals surrounding the subject in a cylindrical shape. By using the information from a pair of photons registered oppose to each other within a coincidence window of ~4 nanoseconds, it is possible to determining the line of response and thereby where in the body the decay took place. A transmission scan is used to create an individual attenuation map which corrects for any radiation that is absorbed by bone and tissue. After further correction of e.g., scattered events and random coincidences, a 3D PET image is obtained. Manual or automatic co-registration of the 3D PET-image with an anatomical T1 weighted Magnetic Resonance Imaging (MRI) image makes it possible to estimate the number of decays in a certain volume of interest. In dynamic PET-scans (scanning over time), the concentration of radioactivity measured as a function of time generates time activity curves which can be used to quantify the binding potential (BP) in a region of interest. BP is the product of receptor density and affinity of the ligand binding [27]. Quantification of a target receptor is performed with kinetic modeling where the concentration of the radioactivity in a region of interest is extracted from the PET data and fitted to a function to estimate the BP. Specific BP in the target tissue can be quantified relative to a reference, which can be either free or total plasma concentration (generating BP_F and BP_P respectively [27]), but the method for deriving these are cumbersome since it requires blood sampling with arterial cannulation. Instead, the simplified reference tissue model (SRTM)[28] can be used, which yields non-displaceable binding potential (BP_{ND}) [27]. BP_{ND} is obtained by using a reference tissue region under the assumption that 1) the reference region has no specific binding (i.e., devoid of target receptors), 2) the target and reference tissue have the same non-displaceable volume of distribution, 3) the kinetic behavior of the radioligand in the target and reference tissue can be modelled by a one tissue compartment model, and 4) the blood volume contribution to both the reference and target tissue is negligible [28,29].

BP_{ND} is defined as:

$$\mathbf{BP_{ND}} = \frac{f_{ND} \times Bavail}{K_D}$$

were f_{ND} is the fraction of free radioligand in the non-displaceable compartment, B_{avail} is the concentration of available receptors and K_D is the radioligand dissociation constant at equilibrium.

PET-studies and 5-HT receptor or transporter alterations in MDD

So far, most clinical PET-studies investigating 5-HT receptor levels in patients with MDD have targeted the 5-HT-$_{1A}$, $_{2A}$ receptor or the SERT. It is widely assumed that 5-HT plays a key role in MDD and the results from these studies have generally shown alterations in the serotonergic system in patients with MDD compared to healthy controls, although in alternating directions. For example, one group has consistently found *higher* 5-HT_{1A} receptor binding in patients with MDD versus healthy controls, both pre-treatment and between episodes [30–32], while others have not been able to replicate these findings [33–35]. Indeed, a large meta-analysis of the 5-HT_{1A} receptor binding in MDD reported *lower* 5-HT_{1A} receptor binding versus healthy controls, but also pointed out that the studies were heterogeneous with varying study-protocols, had different BP outcome and limited sample sizes [36], and some of the studies included cohorts of bipolar and post-partum depression. One study including healthy twins found lower SERT binding in dorsolateral prefrontal cortex in those with high familiar risk for MDD, suggesting a trait marker of MDD [37]. A meta-analysis of *in vivo* and post mortem studies found a decrease in SERT availability in antidepressant-free patients with MDD compared to healthy controls in key regions involved in MDD (brainstem, amygdala and striatum) which could indicate a decrease of 5-HT in MDD [38]. While the authors state that this may support the serotonin hypothesis of a 5-HT decrease in MDD, they did not exclude higher endogenous 5-HT levels in MDD. It could also be argued that low SERT binding indicates an increase in endogenous 5-HT since the reuptake could be decreased with fewer transporters.

PET neuroimaging and antidepressant treatment response in MDD

There are some studies using PET neuroimaging to test for associations to- or prediction of treatment response in MDD. One study found that the cerebral blood flow in the prefrontal cortex measured with single-photon emission computed tomography predicted treatment response after 4 weeks of citalopram treatment [39]. Another group reported that higher pre-treatment SERT binding in diencephalon was associated with better early treatment response at week 4 in a newly recruited cohort with MDD, although the effect disappeared at week 6 [40]. Yet another group found that baseline SERT binding in six different brain regions was borderline predictive of remission status after one year of naturalistic antidepressant treatment (p=0.057), but the cohort was small (n=19), the treatment was not standardized and the study did not adjust for multiple comparison [41]. A few studies have investigated changes in binding after SSRI intervention in depressed patients. At least two studies were not able to find any change of 5-HT_{1A} binding at rescan following SSRI treatment [35,42]. In contrast, five to nine weeks of SSRI treatment in a depressed cohort resulted in an 18% downregulation of the 5-HT_{1A} autoreceptor binding in the raphe, but the reduction was not linked to treatment response [43].

While prediction of antidepressant treatment response based on PET BP measures seems challenging, a potential for distinguishing patients with non-response or response to medicine might be more promising. The pretreatment 5-HT_{1A} receptor binding in the orbitofrontal cortex was found to be higher in non-responders versus responders to SSRI [42]. Another group found an association between higher 5-HT_{1A} receptor binding in the raphe nuclei and being a non-responder after eight weeks of an SSRI treatment when using BP_F (but not BP_{ND}) as outcome measure, though the study was not able to predict treatment response [44]. Yet another study with a larger sample (n=82) found a correlation between brain glucose metabolism and treatment response (non-responder or remitter after 12 weeks escitalopram or cognitive therapy treatment) in six cortical and limbic regions, most pronounced in the right insula [45]. However, the study was later criticized for being underpowered, not presenting any specificity or sensitivity data for implementation to the clinic, and did also not correct for multiple comparisons [46].

Altogether, no robust evidence for serotonergic receptor alterations in MDD has been brought forth, nor convincing or clinically useful studies of prediction of treatment response based on PET measures [47]. Expanding the research to other neuronal networks implicated in MDD has been emphasized [48].

Implications of the 5-HT$_4$ receptor in MDD

The 5-HT$_4$ receptor (5-HT$_4$R) is a member of the larger 7 receptor 5-HT family. It is a G_s-protein-coupled postsynaptic heteroreceptor with downstream cyclic adenosine monophosphate (cAMP) activation and increased neuronal excitability [49]. The amino terminal ends extracellularly and the carboxyl terminal, which is oriented towards the cytoplasm, has at least six known splice variants [50] with yet unclarified distinct functions. The 5-HT$_4$R is widely distributed in the body, both peripherally (mainly in the gastrointestinal tract, heart, bladder and adrenal glands) [51] and in the central nervous system with highest expression found in the basal ganglia (n. caudate, putamen), hippocampal formation (intermediate expression), and neocortex (low expression) whereas cerebellum is devoid of receptors, as found in preclinical- , post mortem- and human *in vivo* imaging studies [50,52–56]. The 5-HT$_4$R is involved in learning, memory, appetite and mood/anxiety disorders [57], and increased focus on the association to MDD has yielded intriguing results from both animal and human studies [50,57]. For example, after short-term administration of 5-HT$_4$R agonists, rodents have shown rapid antidepressant/anxiolytic-like behavior [58,59], prophylactic antidepressant/ anxiolytic properties [60] and hippocampal neurogenesis [61]. A recent first translational study found enhanced memory effects in healthy volunteers after a single dose of the 5-HT$_4$R partial agonist prucalopride, but no antidepressant effects tested with emotion processing tasks [62]. In line with these findings, it has been suggested that 5-HT$_4$R agonists could constitute a new (or add-on) therapeutic target for treatment of MDD and anxiety [58,59,62].

PET-studies and the 5-HT$_4$R

[^{11}C]SB207145 is a PET antagonist radioligand that binds to the 5-HT$_4$R in the brain. The *in vivo* [^{11}C]SB207145 binding corresponds to the known 5-HT$_4$R distribution seen in previous *in vivo* and *in vitro* studies [54,63], and the *in vivo* and *in vitro* receptor density have shown to be significantly correlated ($r=0.86$, $p=0.04$) [64]. The SRTM has been validated as a suitable model for quantification of the 5-HT$_4$R, although a 20-40 % underestimation in high binding regions (putamen and caudate nucleus) compared with using the gold standard two tissue compartment model was observed [53].

A direct effect on the 5-HT_4R from 5-HT modulation has been found in several experimental models. Chronic (but not acute) administration of paroxetine (an SSRI) in rats resulted in a decrease 5-HT_4R binding in a range of brain areas, while 14 and 21 days of 5-HT depletion increased the 5-HT_4R binding [65]. Similarly, two other rodent studies showed a decrease in hippocampal 5-HT_4R binding after chronic fluoxetine (SSRI) or venlafaxine (a serotonin norepinephrine reuptake inhibitor, SNRI) treatment, but not for reboxetine (a norepinephrine reuptake inhibitor) [66,67]. Further, SERT knock out mice had lower levels of 5-HT_4R binding whereas mice overexpressing SERT had higher 5-HT_4R binding [68].

There are only few clinical studies investigating a role of the 5-HT_4R in MDD. One study found higher 5-HT_4R binding and cAMP levels in frontal cortex and caudate nucleus post-mortem in depressed suicide victims compared to healthy individuals [69]. A Japanese case-control study found an association between 5-HT_4R gene polymorphisms and bipolar depression [70]. A previous study of healthy individuals found a negative association between family history of MDD and striatal, but not cortical, 5-HT_4R binding in a dose-dependent manner [71]. This finding supports the involvement of 5-HT_4R in MDD and lower 5-HT_4R availability was speculated to constitute a trait marker for increased risk of MDD. Another clinical study demonstrated that central 5-HT levels can be indexed in an inverse manner through [^{11}C]SB207145 imaging of the 5-HT_4R *in vivo*; a single dose of citalopram did not change the 5-HT_4R binding [64], but three weeks of (randomized) placebo or fluoxetine intervention in 35 healthy participants showed a 5% lower global 5-HT_4R binding in the active group compared to controls [72].

Altogether, both preclinical and clinical findings support an inverse relation between 5-HT_4R levels and central 5-HT availability, which generates a unique possibility to make use of the [^{11}C]SB207145 to quantify 5-HT_4R BP_{ND} per se, but also as an indirect marker for cerebral 5-HT levels in disorders with a serotonergic involvement, e.g. MDD.

Aim

The aim of this PhD study was, for the first time, to study the 5-HT_4R in patients with MDD by using the radioligand [^{11}C]SB207145 and to investigate if alterations in binding were associated with disease status (i.e., MDD or healthy). Also, based on the assumption that 5-HT_4R binding provides an index of the 5-HT tonus [72] we aimed to evaluate [^{11}C]SB207145 binding as a predictor of serotonergic pharmacological treatment response. This also implies a possible stratification of MDD into subtypes based on different [^{11}C]SB207145 binding according to response-profiles. Lastly, we aimed to study changes in [^{11}C]SB207145 binding after 8 weeks of serotonergic antidepressant drug therapy and if an alteration was associated with clinical treatment outcome.

Hypotheses

1. Patients with MDD differ in cerebral [^{11}C]SB207145 binding at baseline compared to healthy controls.
2. [^{11}C]SB207145 binding at baseline in patients with MDD predicts remission after 8 weeks of pharmacological serotonergic intervention.
3. After 8 weeks of serotonergic intervention, patients in remission have a significantly greater reduction in cerebral [^{11}C]SB207145 binding than non-responders.

Description of the research project

This PhD-thesis builds on the main PET trial within the larger initiative "NeuroPharm"-1 (https://np.nru.dk/). The aim of NeuroPharm-1 is to study neurobiological disease mechanisms in the brain and predict treatment efficacy and brain responses to neuro-modulatory interventions, using a range of modalities (PET, Magnetic Resonance Imaging (MRI), electroencephalogram, cognitive testing, questionnaires and peripheral biomaterials). Apart from other large clinical studies with similar scopes (e.g., CAN-BIND [73] and iSPOT-D [74]), the inclusion of an exceptional large PET cohort in our trial allows for studying effects on a neurotransmitter level and thus adds a unique contribution. The study was designed as a naturalistic, open, longitudinal and non-randomized clinical trial including antidepressant-free patients with moderate to severe MDD. Healthy controls were included for comparison of baseline parameters. The naturalistic treatment design was applied in order to mimic and draw conclusions to the real-world clinic (similar to e.g. the STAR*D trial [75]).

Paper I maintains the clinical trial study protocol on which the PET-study in Paper II is based upon. Both papers are presented and discussed in the following of this dissertation. For Paper II, three sections are presented and discussed separately, namely I) Findings from the baseline assessment, II) Findings from the treatment outcomes, and III) Findings from the rescan assessment.

Paper I – Clinical trial study protocol

Study design, recruitment and validation of participants

One hundred patients with MDD were recruited from an out-patient referral center within the Mental Health department, or from collaborating primary care physicians in the capital region of Denmark. Healthy controls were either collected from a pre-existing repository [76] or recruited from online advertisement for the project (www.nru.dk). All healthy controls went through examinations corresponding to the baseline program of the patients. A full list of inclusion and exclusion criteria for all participants is shown in BOX 1. Importantly, all included patients were antidepressant-free at baseline for at least 2 months and had a moderate to severe depressive episode (single or recurrent) according to the ICD-10 diagnostic manual. Hamilton Depression Rating Scale -17 items ($HAMD_{17}$) [77] is an interview-based questionnaire with scores ranging from 0-52 that can be used to assess and monitor the severity of a depressive episode. All patients were recruited face-to-face, had a $HAMD_{17}$ score ≥ 18 corresponding to a severity of at least moderate depression, and were interviewed using the diagnostic Mini International Neuropsychiatric Interview (MINI; version 6.0.0 based on DSM-IV-Text Revision) which covers a range of psychiatric conditions including bipolar disorder. The MDD diagnose was verified by a specialist in psychiatry prior to inclusion in the trial. Healthy controls were screened for current or previous mental health disorders and filled out the Major Depressive Inventory (MDI) questionnaire [78].

Approvals from relevant authorities

All participants signed an informed consent and the trial was approved by relevant authorities: the Committees on Health Research Ethics in the Capital Region of Denmark (ID: H-15017713), the Danish Data Protection Agency (ID: 04711/RH-2016-163) and the Danish Medicines Agency (ID: NeuroPharm-NP1). The study was registered as a clinical trial at clinicaltrials.gov (ID: NCT02869035) and EudraCT (ID: 2016-001626-34) before the first patient was included and a Good Clinical Practice (GCP) unit from the capital region of Denmark monitored the study throughout its active phase.

Baseline examinations

After inclusion, both patients and healthy controls went through a baseline assessment program including medical and psychiatric history, demographic questionnaires, somatic and neurological examinations, urine toxicology and pregnancy test, routine blood tests, genetic status for the serotonin transporter-linked polymorphic region (5-HTTLPR, homozygote for the L_A allele or not, i.e., "L_A/L_A" or "non-L_A/L_A), and a PET and MRI-scan. An electrocardiogram was collected for patients to exclude QTc prolongation (which would contraindicate treatment with the trial-drug).

PATIENTS WITH MDD

Inclusion criteria

- Age 18-65
- Moderate to severe depression ($HAMD_{17} \geq 18$)
- No antidepressant treatment within the last 2 months
- Current depression < 2 years duration
- No other primary psychiatric condition

Exclusion criteria

- Psychotic
- Acute suicidal ideations
- Alcohol use disorder
- Substance use disorder/use of substances within the last month
- Previous non-response to SSRI
- Indication for other treatment than SSRI
- Any contraindication to the study protocol
- Severe somatic co-morbidity
- Medicine that might influence trial
- Pregnancy or breast-feeding
- Insufficient comprehensiveness to Danish tasks
- Previous severe head trauma
- Exposure to radioactivity of > 10 mSv within the last year

HEALTHY CONTROLS

Inclusion criteria

- Age 18-65
- No previous or current mental disorder

Exclusion criteria

- Alcohol use disorder
- Substance use disorder or use of substances within the last month
- Any contraindication to the study protocol
- Somatic condition/medicine that might influence the trial
- Pregnancy or breast-feeding
- Insufficient comprehensiveness to Danish tasks
- Previous severe head trauma
- Exposure to radioactivity of > 10 mSv within the last year

BOX 1. Inclusion and exclusion criteria for patients with MDD and healthy controls

Follow-up assessments

After completion of the baseline program, patients started antidepressant treatment with the SSRI escitalopram: 5 mg for three to five days to avoid excessive side effects during the initial phase, followed by a daily dose of 10 mg. Adjustments of the dose ranged between 10-20 mg daily depending on treatment response and side effects. No concurrent treatment (e.g. psychotherapy or other antidepressant medication) was allowed during the study. Clinical follow-up visits were scheduled after 1, 2, 4, 8 and 12 weeks of treatment and carried out by a study physician or study assistant supervised by a specialist in psychiatry. The visits were strictly focused on the medical treatment to avoid any therapeutic alliance. The clinical treatment response was monitored at each visit by using the $HAMD_{17}$ and the subscale of 6 items ($HAMD_6$, ranging from 0-22) which has proven to be a reliable measure of changes in MDD core symptoms [79,80]. Figure 1 illustrates a simplified overview of the study program. Side effects were monitored at each follow-up with the "UKU" questionnaire [81]. Compliance to the medicine was controlled by pill-count at each visit and collection of trough serum drug concentrations at week 8. The clinical visits could deviate one week from the original time schedule, i.e. a visit at week 4 could be held within ±6 days from week 4. Extra visits were allowed if clinically motivated. When necessary, add-on of sleeping pills (non-benzodiazepine sedatives) and to a lesser extent anxiolytics (benzodiazepines) to reduce initial side effects to the treatment was allowed, but patients were asked to avoid usage within 72 h from PET-scan days. Monthly co-ratings for calibration of study-investigators involved in $HAMD_{17/6}$-ratings were implemented and the results of these were controlled by the study group and by the monitoring GCP unit. The limit for an acceptable deviation in total $HAMD_{17/6}$ score between raters and the chief psychiatrist (i.e., the reference) was set to ± 20%; a difference exceeding this required a new satisfactory co-rating before any new data collection was allowed from that particular rater.

At the week 4 visit at the earliest, a switch to duloxetine (an SNRI) was offered, but not mandatory, to patients with adverse side effects or a clinical response of <25 % change in $HAMD_6$ from baseline (i.e., an early non-responder, see definition below). Duloxetine was chosen in accordance with national guidelines for a second line antidepressant drug and because of its low affinity to the 5-HT_4R [82]. The week 4 time point was chosen in order to identify a biological response (or non-response) to SSRI treatment at an early stage and is also in line with treatment guidelines for an adequate trial (4-8 weeks) [83].

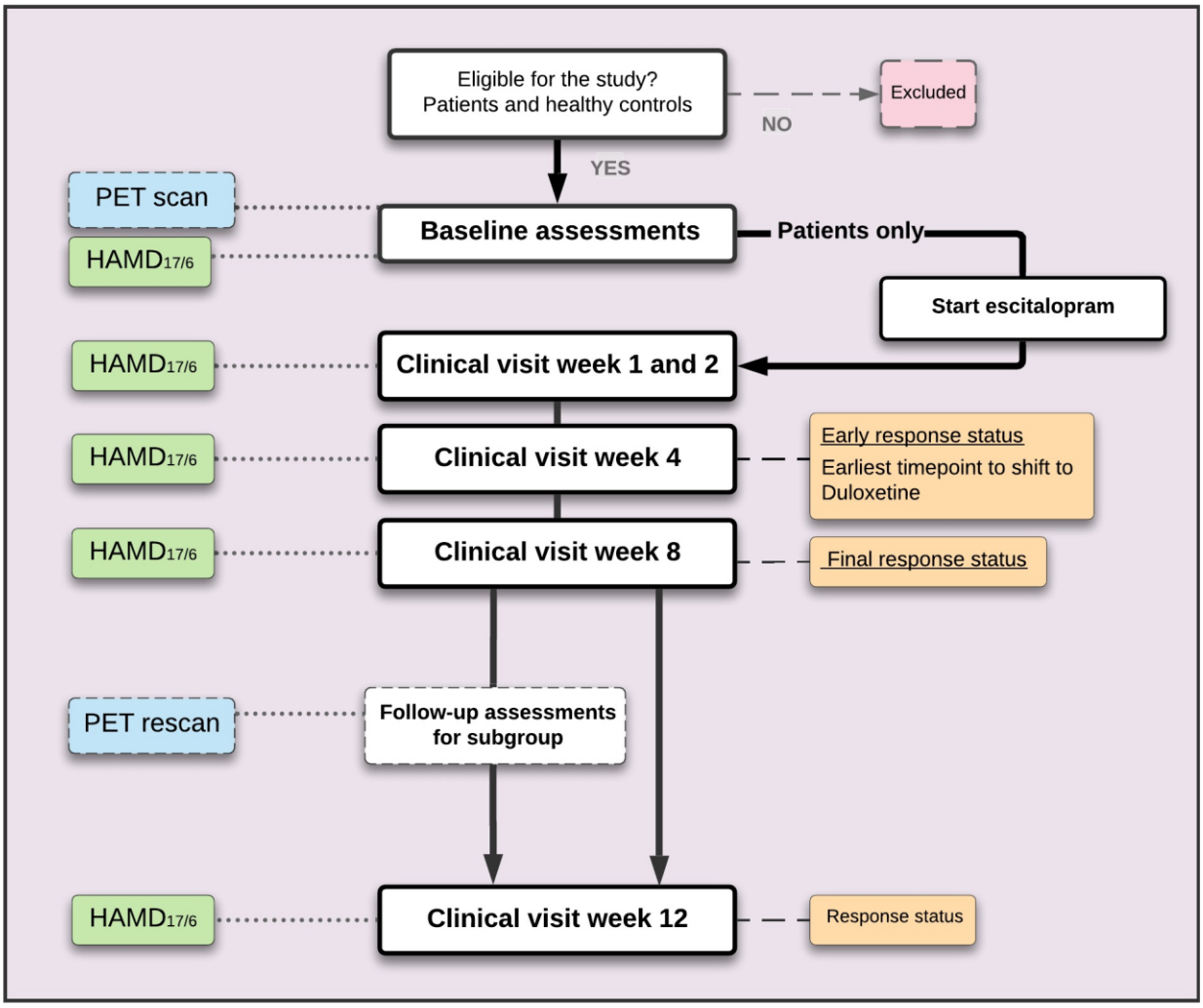


Figure 1. Simplified flowchart of the study program. Patients and healthy controls went through the baseline program including PET scanning. Patients continued with the study program including treatment with escitalopram/duloxetine, clinical visits at week 1, 2, 4, 8 and 12, and monitoring of treatment response with $HAMD_6$. A subgroup of patients was PET rescanned after 8 weeks of treatment. PET: Positron Emission Tomography. $HAMD_{17/6}$: Hamilton Depression Rating Scale 17 and 6 items. Modified from Paper I (Köhler-Forsberg et al 2020, *in review*).

Rescan assessment

After 8 weeks of treatment, a subgroup of patients (n=43) received a PET and MRI rescan. All patients (independent of response status) were offered the rescan examination in a continuous fashion until allotted scans were completed.

Primary outcome measure – categorical clinical response

The primary outcome measure was clinical response status at week 8 where patients were categorized based on a percentage change in $HAMD_6$ scores from baseline (Figure 2). Remitters had to meet the criteria for early response at week 4 (≥50% reduction from baseline in $HAMD_6$) and have a final $HAMD_6$ <5 at week 8. Non-responders had to meet the criteria for early non-response at week 4 (<25% reduction from baseline in $HAMD_6$) and have <50% reduction in $HAMD_6$ at week 8. If a patient did not categorize as an early responder or early non-responder at week 4, they were defined as "other", and if they did not categorize as remitter or non-responder at week 8, they were defined as intermediate responder. See Figure 2 for a schematic overview.

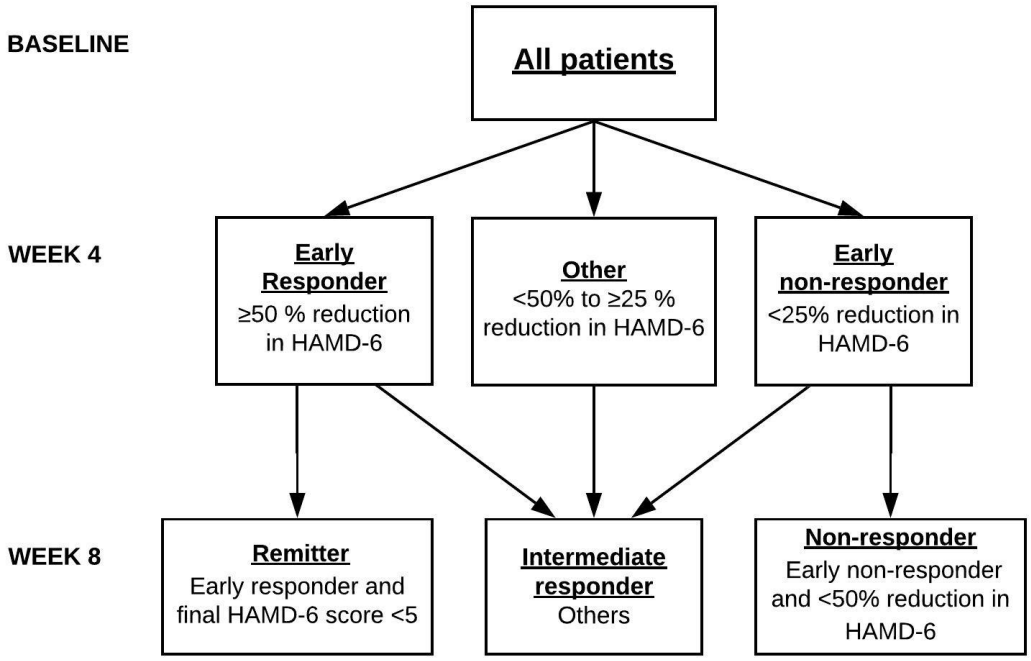


Figure 2. Primary outcome measure. Categorical response status at week 4 and 8, based on changes in baseline $HAMD_6$ score.

Secondary outcome measure – continuous clinical response

The secondary clinical outcome measure, $r\Delta HAMD_6$, was based on percentage changes in $HAMD_6$ from baseline in a continuous fashion instead of a categorical approach, in order to test for an association between continuous treatment response and baseline 5-HT_4R BP_{ND} or treatment-induced changes in BP_{ND}.

$$r\Delta HAMD_{6=}\frac{HAMD6(week\ x)-HAMD6(baseline)}{HAMD6(baseline)}$$

PET imaging, MRI acquisition and data preprocessing

Dynamic PET scans were acquired with a 3D high-resolution tomography Siemens PET scanner (CTI/Siemens, Knoxville, TN, USA) (image matrix: 256 × 256 × 207 voxels; 1.22 × 1.22 × 1.22 mm) with a resolution down to 1.5 mm in the center of field of view [84]. First, a 6 min transmission scan was performed for attenuation correction. An intravenous bolus injection of [^{11}C]SB207145 targeting 600 MBq was then given over 20 seconds followed by a 120 minutes PET scan. The [^{11}C]SB207145 was automatically produced in a local nuclear medicine facility immediately before injection, as previously described [53]. The injection of [^{11}C]SB207145 did not cause any adverse events. Participants were scanned in a supine position with an in-house developed head holder to reduce movements during the scan. The scan was reconstructed into 38 frames (6x5 s, 10x15 s, 4x30 s, 5x2 min, 5x5 min, and 8x10 min) with a 3D-OSEM PSF algorithm (16 subsets, 10 iterations) and TXTV-based attenuation correction was applied [85]. Air 5.2.5 [86] was used for correction of intra-scan movement, aligning each participant's PET frame to the first five-minute frame. High-resolution structural T1 weighted MRI scans were obtained from all participants. A medical specialist in neuroradiology searched all patient MRI scans for pathology. All patients and 53 healthy controls were MRI-scanned with a Siemens Prisma 3-Tesla scanner (Erlangen, Germany) with a 64-channel head coil. Because of inclusion of historic controls from the repository, 38 healthy controls had previously been MR-scanned with a Siemens Magnetom Trio 3-Tesla scanner. All MRI images were segmented into cerebrospinal fluid and grey- and white matter. PET images and the corresponding T1 weighted MRI image were aligned and co-registered with SPM8. The Pvelab software package [87] was used for extraction of regions of interest which were automatically delineated on each individuals' MRI based on a verified probability-map. Correct segmentation, co-registration in three planes and placement of regions of interest were visually controlled by a trained study investigator. Mean time activity curves for volume-weighted sums of left and right grey matter regions were extracted for kinetic modeling. The SRTM with cerebellum (excluding vermis) as reference region (previously validated for [^{11}C]SB207145 [53]) was used in the kinetic modeling, yielding BP_{ND} as the outcome measure for the 5-HT_4R binding.

Statistical analyses

One hundred patients were included in order to obtain a power of 80%. The calculation was based on the assumptions of a 20% drop-out rate, 50% remission rate at week 8, and a minimum of 7% difference in 5-HT_4R binding between remitters and non-responders (built on a previous observed variation depending on the 5-HTTPLR gene status [88]). Group differences in the descriptive tables were tested by using Fisher's exact t-test for categorical variables, and Mann Whitney U-test for continuous variables. Patients were matched with healthy controls based on age and sex to reach roughly the same distribution. Standard diagnostic tools were used for validation of normality assumptions, e.g., quantile-quantile-plots were generated and inspected for discrepancies and if necessary, a non-parametric bootstrap was used to assess the robustness of the p-value to the normality assumption. When finding statistically significant effects, we checked that no single observation had a disproportionate influence on the estimated effect. All binding potential values were log-transformed to better match normality assumptions and regional effects are reported after back-transformation. Because of their influence on the [^{11}C]SB207145 binding, analyses were adjusted for age, sex [89], injected tracer (mass/kg) [90], and the 5-HTTLPR polymorphism genotype (L_A/L_A or non-L_A/L_A) [88,91]. When healthy controls were included, the analyses were also adjusted for MRI-scanner type. For the rescan analyses, we only adjusted for injected tracer (mass/kg) since the other covariates were static.

Longitudinal data analyses only included patients with a documented compliance to the study protocol at week 8. Missing data at baseline was handled as missing completely at random. For the primary longitudinal analyses, missing data from 9 patients was imputed based on the week 4 response (when available): patients leaving the study because of early remission were ascribed as remitters and those leaving due to suicidality or adverse side effects were ascribed as non-responders. Other types of drop-out were handled with inverse probability weighting by using baseline covariates (severity of the depressive episode and sex) as predictors of drop-out. For the secondary analyses, we used a complete case analysis approach.

Patients with serum drug tests below the detectable level at week 8 (escitalopram <10 nM; duloxetine <15 nM), who missed their week 8 visit or who had taken <2/3 of their prescribed trial-drug were not included in the longitudinal analyses since they were not considered to have a documented compliance to the study protocol. Two-sided p-values < 0.05 were considered

statistically significant, and adjusted p-values are denoted as “p.adj”. All statistical analyses were performed in R.

Primary analyses

As the primary analysis model, we used a latent variable model (LVM) to test for global and regional differences between groups across the primary regions of interest. LVM is a statistical model which can utilize the observed 5-HT_4R BP_{ND} in multiple brain regions and summarize them into a latent variable, e.g. a difference in 5-HT_4R BP_{ND} across brain regions. The model can then be used to test for a global effect, i.e. is there an association in any brain region, but also to display region-specific effects. Since LVM can test multiple regions at the same time, adjustments for multiple comparison is unnecessary at a global level. The regions of interest were 1) neocortex (a larger region modelled based on combined time activity curves: orbitofrontal cortex, middle inferior frontal gyrus, medial inferior temporal gyrus, superior frontal gyrus, parietal cortex, occipital cortex, superior temporal gyrus and sensory motor cortex), 2) hippocampus, 3) caudate nucleus and 4) putamen, see Figure 3 for a representative image. These regions were primarily chosen to represent large and wide-spread brain regions for detection of changes in global 5-HT tonus, but also based on their involvement in MDD and previous work on the 5-HT_4R from our group [56,71,72,91]. Especially caudate nucleus and putamen have high binding properties of [^{11}C]SB207145 and a good signal-to-noise ratio, and hippocampus is densely innervated by 5-HT [92]. Neocortex was chosen to give a robust and wide-spread binding measurement despite being a low-binding region for [^{11}C]SB207145.

In the primary analyses, we tested both (A) for a difference in 5-HT_4R BP_{ND} between patients with MDD and healthy controls at baseline, and (B) baseline 5-HT_4R BP_{ND} differences between non-responders, remitters and healthy controls tested against each other. Further, we tested for (C) a change in BP_{ND} from baseline to rescan (ΔBP_{ND}), including (D) if a change in binding was associated with non-responder/remitter status at week 8. To (E) assess if the 5-HT_4R BP_{ND} at baseline could discriminate non-responders from remitters at week 8, we used a receiver operating characteristics (ROC) curve. Individual ROC curves were created for each brain region displaying the sensitivity and specificity across various thresholds of the regional 5-HT_4R BP_{ND}. The area under the ROC curve (AUC) was calculated to summarize the predictive performance of the baseline 5-HT_4R BP_{ND}, where a value of 0.5 indicates an uninformative classifier and 1 is perfect discrimination.

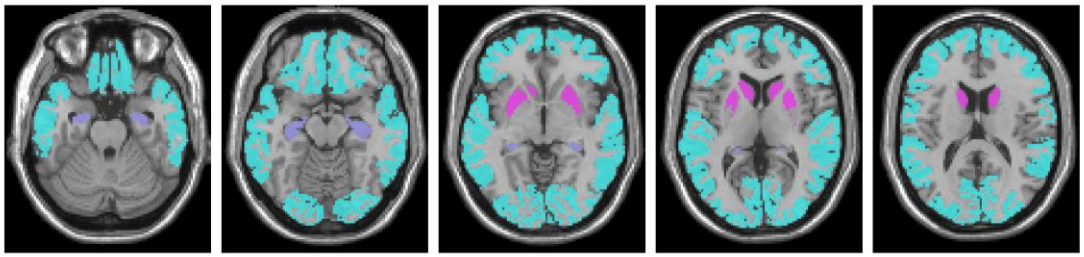


Figure 3. **Representative image of the regions of interest**. Neocortex (turquoise), hippocampus (violet/blue) and neostriatum (pink).

Secondary analyses

In the secondary analyses, we used the primary analysis model LVM to test for (a) an association between continuous response ($r\Delta HAMD_6$) for all patients and baseline 5-HT_4R BP_{ND}, (b) baseline 5-HT_4R BP_{ND} differences between early non-responders, early responders and healthy controls, and finally (c) an association between $r\Delta HAMD_6$ and ΔBP_{ND}. We also tested for (d) the predictive value of baseline 5-HT_4R BP_{ND} to determine early response/early non-response at week 4.

For a more standardized statistical approach, we used multiple linear regression for (A), (B), (a) and (b) where we tested three pooled brain regions independently: a) neostriatum (putamen and caudate nucleus), b) neocortex and c) a limbic region (hippocampus, amygdala, thalamus, anterior cingulate gyrus, posterior cingulate gyrus) [87].

Paper II

I. Findings from the baseline assessment

Patient characteristics at baseline

Characteristics of patients and healthy controls are presented in Table 1. There were no differences between groups except for years of education, MDI score and injected tracer (mass/kg). The difference between groups for the MDI score was expected and (in addition to the screening at the recruitment) verifies the control group as not being depressed. The difference between groups in years of education was statistically significant, but not evident in terms of mean-values (11.6 years vs 11.9 years for patients and healthy controls respectively). It has been shown that lower education is associated with risk for MDD [93], and as such, an even greater difference would not have been surprising. The injected tracer (mass/kg) was significantly higher in healthy controls than in depressed patients. For [^{11}C]SB207145, injection of high mass of non-labeled (cold) compound can result in underestimation of the binding because of a competitive binding between non-labeled and radiolabeled ligand [90]. A lower 5-HT$_4$R BP_{ND} in patients compared to healthy controls would therefore not likely be explained by lower injected tracer (mass/kg) in patients. Regardless, injected tracer (mass/kg) was used as a covariate in all analyses.

Missing PET-data from 9 patients was handled with a missing completely at random approach: (n=1) acute suicidal ideation; (n=2) excessive anxiety not compatible with scanning procedure; (n=1) tracer production failure; (n=2) unexpected pregnancy discovered at site; (n=2) withdrawal of consent (preferred psychotherapy). In addition, 1 patient underwent the baseline PET scan but was excluded from the analysis because of spontaneous remission without treatment before the week 1 visit. At baseline, 59 patients had a $HAMD_{17}$ score between 18-24 (corresponding to moderate MDD), and 32 patients had a $HAMD_{17}$ score ≥25 (corresponding to severe MDD) (Figure 4).

		Patients with MDD			Healthy controls		
		n	**%**		**n**	**%**	**p-value** [a]
Sex	Female	65	71.4		55	60.4	0.16
	Male	26	28.6		36	39.6	
5-HTTLPR genotype	L_AL_A	26	28.6		27	29.7	1
	Non-L_AL_A	65	71.4		64	70.3	
	Range	**n**	**Mean (SD)**	**Range**	**n**	**Mean (SD)**	**p-value** [b]
Age (years)	18.3-57.3	91	27.1 (8.2)	19.2-60.1	91	27.1 ± 8.0	0.57
Years of education	5-12	76	11.6 (1.1)	9-12	91	11.9 (0.5)	**0.003**
BMI (kg/m^2)	17.1-45.1	91	24.5 (5.6)	18.3-36.9	91	23.6 (3.1)	0.96
$HAMD_{17}$	18-31	91	22.9 (3.4)	NA		NA	NA
$HAMD_6$	7-17	91	12.3 (1.6)	NA		NA	NA
MDI	16-50	89	34.7 (7.2)	0-18	91	5.6 (4.2)	**< 0.001**
Injected dose (MBq)	263.0-615.0	91	577.4 (56.0)	226-617	91	569.4 (76.3)	0.20
Injected mass/kg (µg/kg)	0.004-0.082	91	0.013 (0.015)	0.003-0.07	91	0.017 (0.015)	**0.028**
Cerebellum, area under curve (kBq/ml)	3.9-17.6	91	10.3 (2.6)	3.2-16.2	85	10.3 (2.5)	0.75

Table 1. **Clinical profile, demographic and tracer data for patients with MDD and healthy controls.** BMI: body mass index. $HAMD_{17/6}$: Hamilton depression rating scale 17 or 6 items. MDI: Major depressive inventory. NA: not applicable. [a] p-value computed using a Fisher's exact t-test, [b] p-value computed using a Mann Whitney U-test. The table is adapted from Paper II (Köhler-Forsberg et al 2020, unpublished).

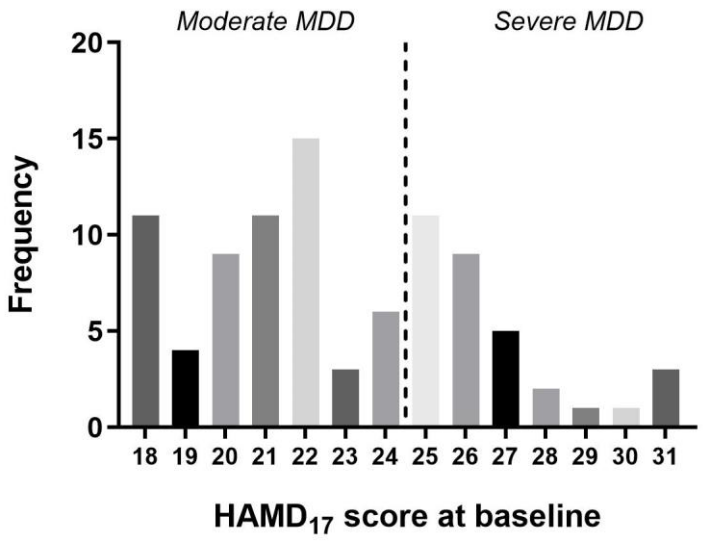


Figure 4. Scatterplot of frequency in $HAMD_{17}$ scores at baseline.

Differences in 5-HT_4R binding between MDD and healthy controls at baseline

We found a significant effect for a difference in global 5-HT_4R BP_{ND} between patients with MDD and healthy controls, tested with a latent variable (γ= -0.08, 95% confidence interval (CI) [-0.1 to -0.03], p=.0009), γ is the effect of group-status on the average binding. At a regional level, there was between 7-8 % lower binding across the four brain regions (Figure 5). Because of the additional strong correlation between caudate nucleus and putamen (visualized as a dashed line in figure 5), we chose to pool these regions into "neostriatum" for all subsequent analyses. The multiple linear regression analysis gave similar results when testing for regional percentage differences in 5-HT_4R binding in MDD versus healthy controls: neocortex (-8.86%, 95%[-14.0 to -3.4], p.adj<0.001), limbic region (-6.53%, 95%CI[-11.4 to -1.4], p.aj<0.01), neostriatum (-6.2%, 95%CI[-11.2 to -1.0], p.adj=0.017).

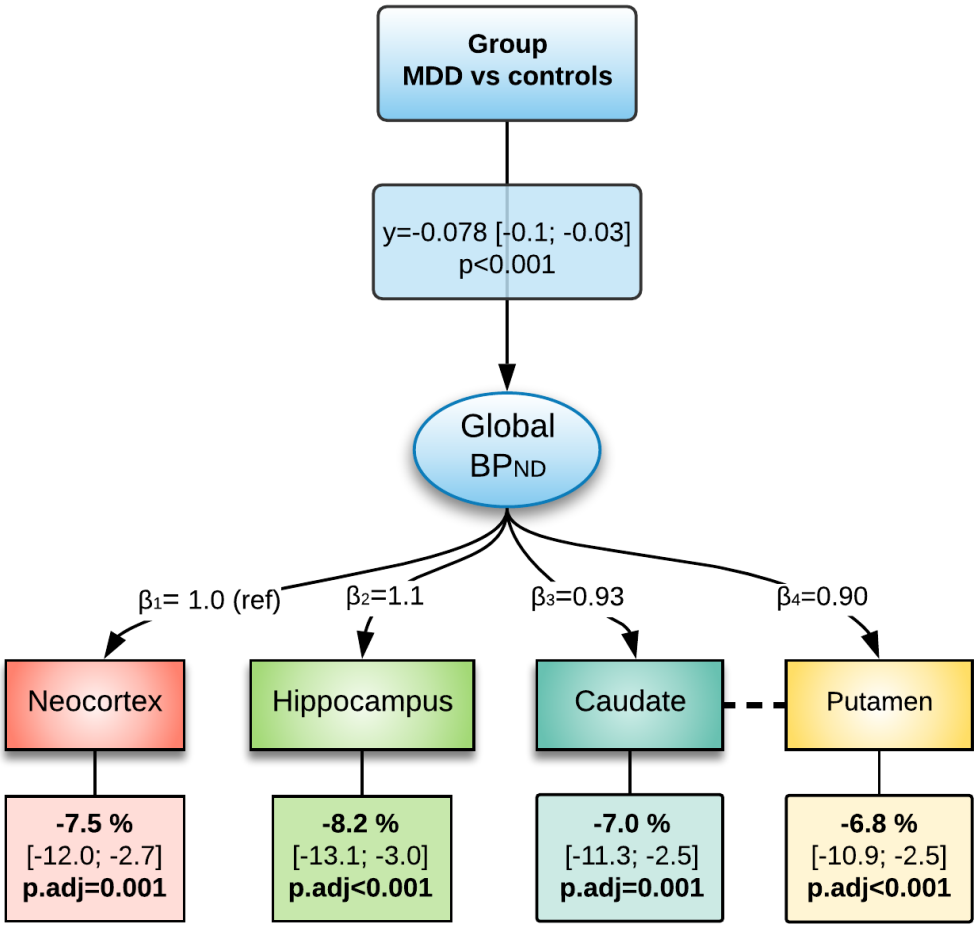


Figure 5. Differences in baseline 5-HT$_4$R BP$_{ND}$ in patients with MDD and healthy controls using the latent variable model. γ represents the effect of group-status on the mean 5-HT$_4$R binding, β is the loading for each region, the dashed bold line shows extra shared correlations between putamen and caudate. The lower boxes indicate the difference between untreated patients with MDD and healthy controls. Confidence intervals and p-values are adjusted. Covariates are not shown for simplicity: age, sex, 5-HTTLPR gene-status, MR-scanner type and injected mass (mass/kg). Figure and caption adapted and modified from Paper II (Köhler-Forsberg et al 2020, unpublished).

Discussion of the baseline findings

We found that antidepressant-free patients with moderate to severe MDD had 7-8% lower cerebral 5-HT$_4$R binding compared to healthy controls across all tested brain regions. Interestingly, a negative association between the number of first degree relatives with a history of depression and striatal 5-HT$_4$R binding has been found in (yet) healthy volunteers [71], suggesting an involvement of the 5-HT$_4$R in MDD where low levels could be a trait marker in a population at-risk who might downregulate the 5-HT$_4$R levels perhaps as a protective or compensatory mechanism to counteract a depressive predisposition. In that sense, lower 5-HT$_4$R

levels in patients with MDD might represent a similar trait (or state) marker. Given the preclinical and clinical evidence of an inverse relation between 5-HT_4R binding and cerebral 5-HT levels [65,66,68,72], our data suggests that patients with MDD have increased cerebral 5-HT levels pre-treatment. Low 5-HT_4R levels might be an attempt to increase synaptic 5-HT in order to remain in a euthymic state. In combination or independently, the low 5-HT_4R binding could also reflect reduced capacity of 5-HT_4R agonism in patients with MDD.

As BP_{ND} is proportional to receptor density and affinity, these factors could contribute to the difference in binding, but since affinity is not likely to differ between patients and healthy controls, alterations in receptor density is the most likely explanation for the observed group differences. Hypothetically, [^{11}C]SB207145 could also cross over the cell membrane and gain access to e.g., internalized receptors. Thus, receptors that are not physiological active could also render PET signals, although the magnitude of such a signal would be difficult to assess, and either way, difference in 5-HT_4R binding between groups was observed.

In contrast to our findings, another study found *higher* 5-HT_4R binding and levels of the second messenger cAMP in frontal cortex and caudate nucleus (but not hippocampus and amygdala) in depressed, violent suicide victims [69]. However, the study was smaller in size (N=19), did not exclude antidepressant medication prior to the suicide (other than tricyclic antidepressants), and had no information about general clinical characterizations, e.g., severity or psychotic symptoms. As such, the studies are not directly comparable. Additionally, receptor binding post mortem and *in vivo* might not coincide, as seen previously [38].

II. Findings from the treatment outcomes

Study outcome over time

Study outcomes for patients are shown in Figure 6. For a more detailed CONSORT flow diagram, see Figure 2 in Paper I. Patient characteristics according to categorical response status at week 8 are shown in Table 2. There was no difference between non-responders and remitters for any variable except for changes in $HAMD_6$. At week 8, at total of 78 patients (13 non-responders (16.7%), 43 intermediate responders (55.1%) and 22 remitters (28.2%)) had been compliant to the medication as measured by blood tests and pill-count, and were included in the longitudinal analyses. Reasons for missing data from nine patients between baseline and week 8

were: spontaneous remission without treatment (n=1), admission to psychiatric hospital due to psychosis (n=1), intolerable side effects to both trial-drugs (n=1), self-reported non-compliance to medicine (n=1), and drop-out without any given reason (n=5). In addition, four patients were excluded from the longitudinal analyses because of undetectable serum-drug level at week 8. Of the patients included in the longitudinal analyses, six switched to duloxetine before week 8, and seven switched after week 8. One patient obtained remission on a dose of 5 mg escitalopram daily (kept low due to side effects), with a serum-escitalopram of 42.48 nM at week 8 which was within one standard deviation from the mean of the group.

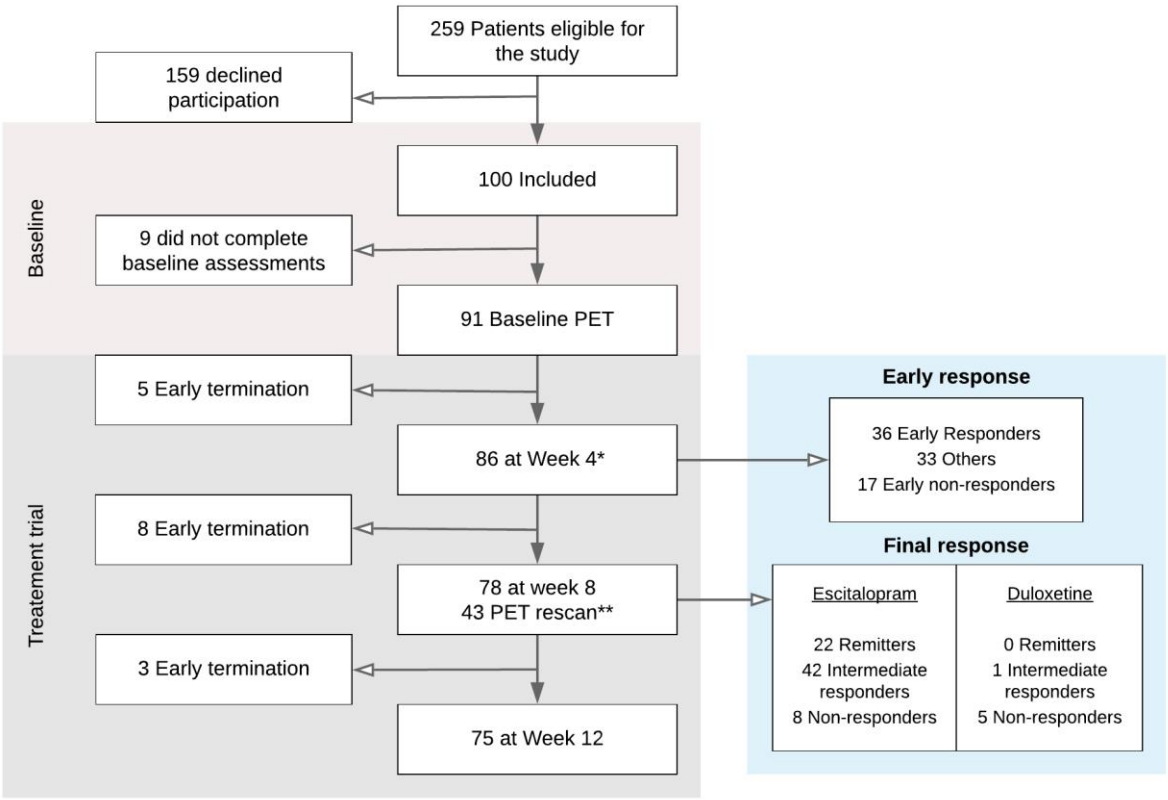


Figure 6. Study outcome for patients. *Only the 78 patients with documented compliance at week 8 were included in analyses for week 4. **43 patients received a PET rescan at week 8, but two patients failed documented compliance at week 8, and 1 patient had an unsuccessful PET-scan (data-failure), resulting in 40 patients included in the rescan analysis.

The remission rate after 8 weeks of treatment was 28.2%, which cannot be directly compared with e.g. Level 1 of STAR*D because of different response criteria and treatment duration. If applying the same criteria (final $HAMD_{17} \leq 7$ at week 12), our remission rate was higher (48%) compared to STAR*D (28%) [24]. However, a meta-analysis of 37 randomized controlled trials with comparable treatment and duration to the STAR*D, found that the average remission rate

was 44% [94], which is more in line with our result. Figure 7 shows changes in mean $HAMD_6$ over time.

	Non-responders (n=13)	Intermediate responders (n=43)	Remitters (n=22)	Non-responders versus remitters p-value [a]
Females, n (%)	10 (77)	34 (79)	13 (59)	0.46
Single/recurrent episode				
Single (n)	5	19	8	
Recurrent (n)	8	24	14	
	Mean (SD)	***Mean (SD)***	***Mean (SD)***	**p-value [b]**
Age	25.9 (10)	26.5 (7.0)	29.1 (9.6)	0.19
Years of education	11.7 (0.6)	11.3 (1.4)	12.0 (0.23)	0.34
$HAMD_{17}$ baseline	21.5 (2.2)	23.1 (3.7)	22.9 (3.0)	0.23
$HAMD_6$ baseline	11.2 (1.8)	12.7 (1.4)	11.9 (1.5)	0.18
%change $HAMD_6$ week 2	8.8 (33.4)	-23.4 (19.5)	-34.6(23.2)	**<0.01**
%change $HAMD_6$ week 4	-0.6 (21.4)	-42.4 (14.4)	-62.3(13.8)	**<0.01**
%change $HAMD_6$ week 8	-9.5 (15.3)	-50.5 (24.7)	-81 (11.6)	**<0.01**
%change $HAMD_6$ week 12	-28.9 (36.7)	-58.6 (26.2)	-85 (11.6)	**<0.01**
MDI baseline	35.9 (5.9)	35.1 (6.9)	32.9 (9.0)	0.19
Dose week 8 (mg)				
-Escitalopram	15 (3.8), n=6[#]	17.7 (3.4), n=42*	15.9 (4.5), n=22	0.50
-Duloxetine	66 (13.4) n=5	60 (NA) n=1	NA	NA
Serum drug week 8 (nM)				
- Escitalopram	85.6 (45.9) n=6[#]	90.4 (49.0) n=42*	60.5 (24.0) n=22	0.28
- Duloxetine	134.0 (89.4) n=5	91.4 (NA) n=1	NA	NA
Injected mass/kg	0.01 (0.02)	0.01 (0.01)	0.01 (0.02)	0.35
Cerebellum, area under curve (kBq/ml)	10.7 (2.5)	10.5 (2.9)	94.4(1.7)	0.11

Table 2. Clinical and demographic characteristics and PET parameters at baseline for patients according to response group at week 8. $HAMD_{17/6}$: Hamilton depression rating scale 17 or 6 items. MDI: Major depressive inventory. NA: Not applicable. Missing observations: [#] n=1 invalid test, n=1 missing test. * n=1 missing test. [a] Fisher's exact t-test, [b] Mann Whitney U-test.

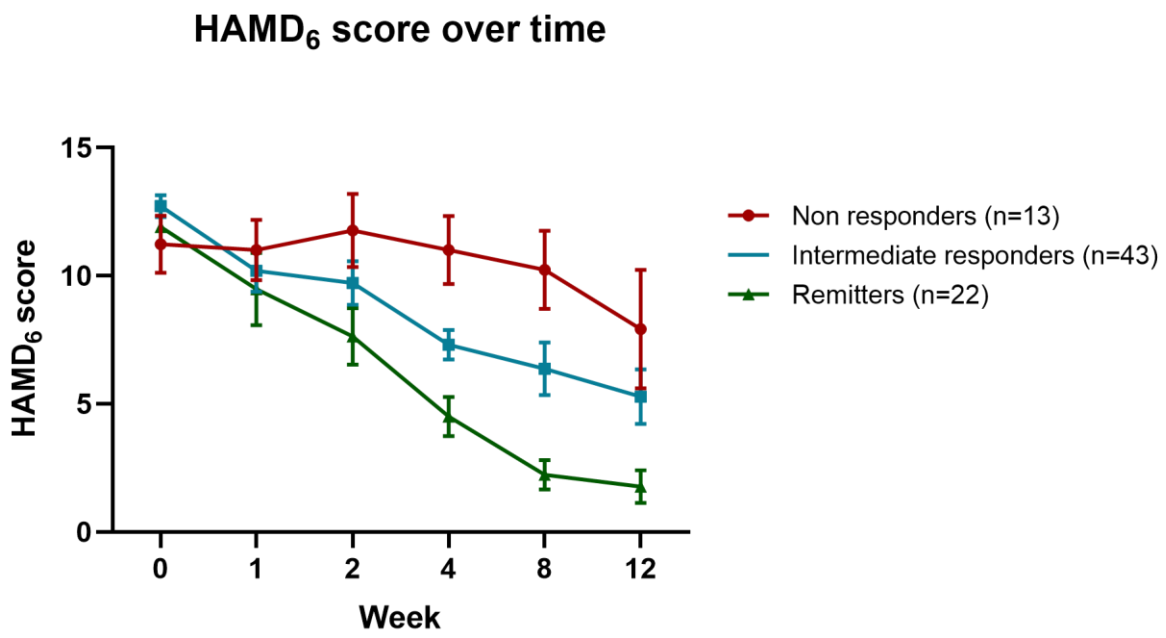


Figure 7. HAMD$_6$ scores according to categorical treatment outcome from baseline to week 12. Mean values for each group are plotted and 95% CI are shown as vertical bars.

5-HT$_4$R binding and the association to antidepressant treatment outcome

We found a decrease in baseline 5-HT$_4$R BP_{ND} at a global level in remitters versus healthy controls (γ= -0.01, 95% CI [-0.17 to -0.03], p=0.0038). At a regional level, there was a decrease of 8-10% across all three brain regions (Figure 8). On the contrary, we found no evidence for differences in 5-HT$_4$R binding between non-responders and healthy controls (p=0.31), or non-responders and remitters (p=0.18). When we included early response (week 4), we found similar results of 8-10% lower baseline 5-HT$_4$R binding in early responders versus healthy controls (global effect p=0.0021; neocortex (-9.0% [-14.6 to -2.9]), hippocampus (-10.0% [-16.3 to -3.3]), neostriatum (-7.7% [-12.6 to -2.5]) and border significance between early non-responders versus early responders (global effect p=0.046; neocortex (-8.0% [-15.6 to 0.27]), hippocampus (-8.9% [-17.3 to 0.31]), neostriatum (-6.9% [-13.5 to 0.23]). See Figure 9 for a representative image of regional differences for neocortex tested with the LVM. We found no difference in 5-HT$_4$R binding at a global level between early non-responders and healthy controls (p=0.79).

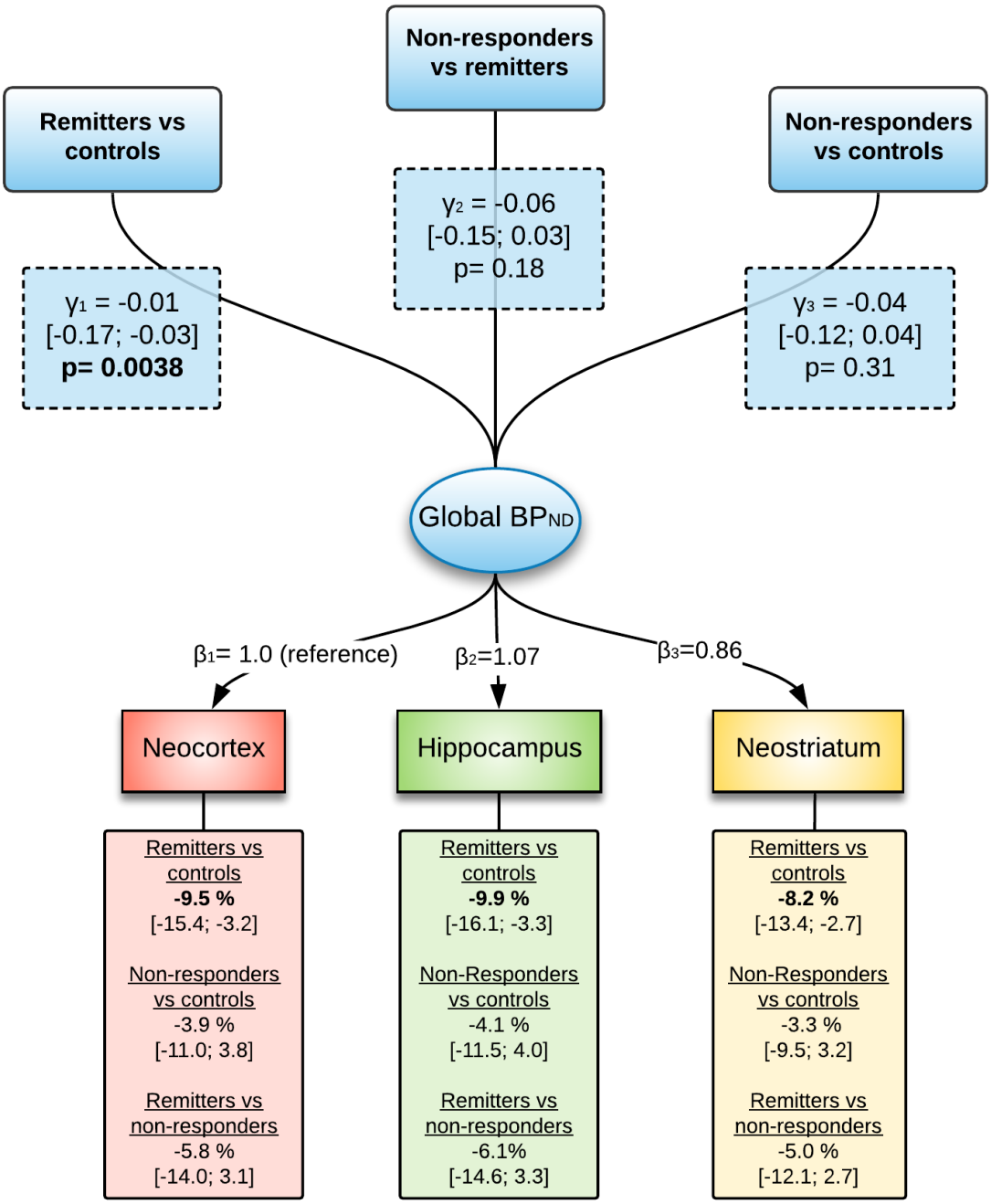


Figure 8. Latent variable model showing differences in baseline 5-HT$_4$R binding between healthy controls and patients with MDD. γ represents the effect of response-status on the global 5-HT$_4$R BP$_{ND}$, β is the loading for each region. The percentage difference in 5-HT$_4$R BP$_{ND}$ between remitters, non-responders and healthy controls are displayed in the lower boxes. Covariates: age, sex, 5-HTTLPR gene-status, MR-scanner type and injected tracer (mass/kg). Figure and caption are adapted and modified from Paper II (Köhler-Forsberg et al 2020, unpublished).

In the multiple linear regression analysis for the categorical outcome, we found a similar neocortical decrease in baseline 5-HT_4R binding among remitters (-8.5% [-16.4; 0.08], p.adj =0.052) and early responders (-8.0% [-14.5; -0.9], p.adj=0.0025) compared with healthy controls, but not for any other clinical outcome or brain region (all p>0.05). Further, we only found a correlation between baseline BP_{ND} and r$\Delta HAMD_6$ at week 4 (r=0.31 [0.09; 0.52], p.adj=0.010) although the clinical relevance of this single finding is limited since the partial correlation coefficient was low. Sensitivity analyses showed that the missing data did not significantly alter our primary findings. In addition, no single observation had a significant impact on the primary results.

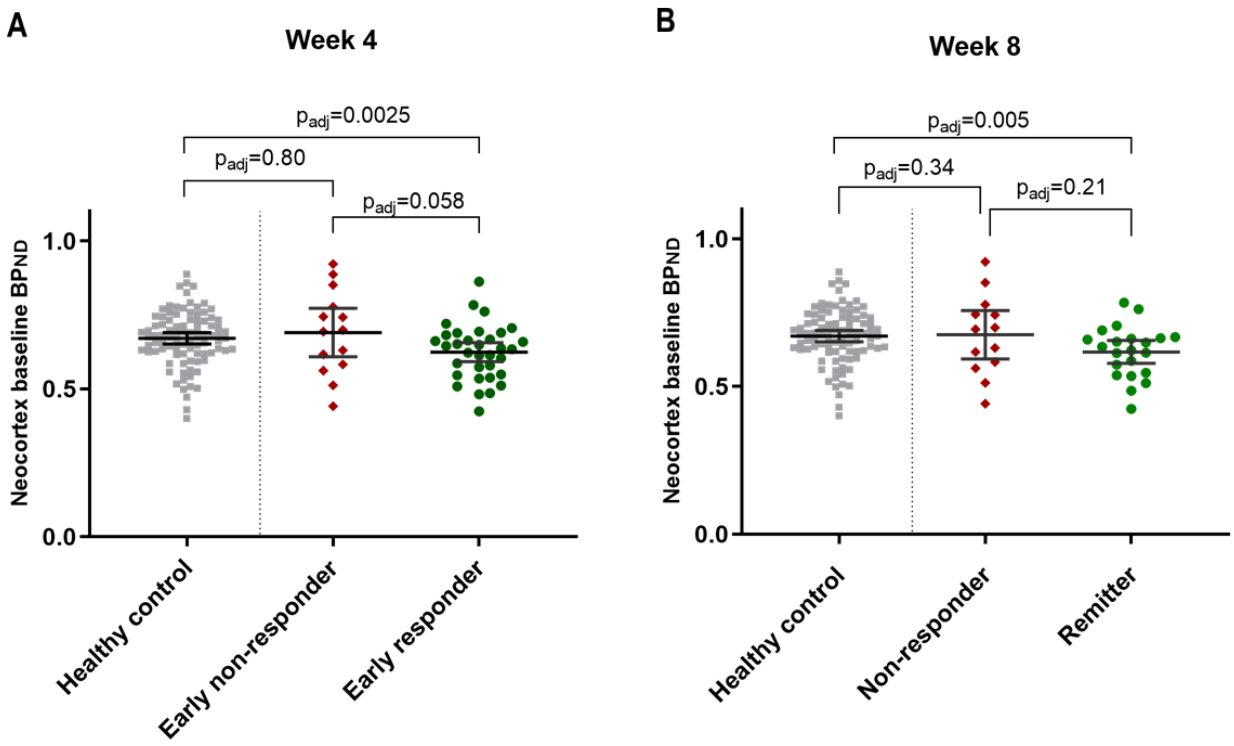


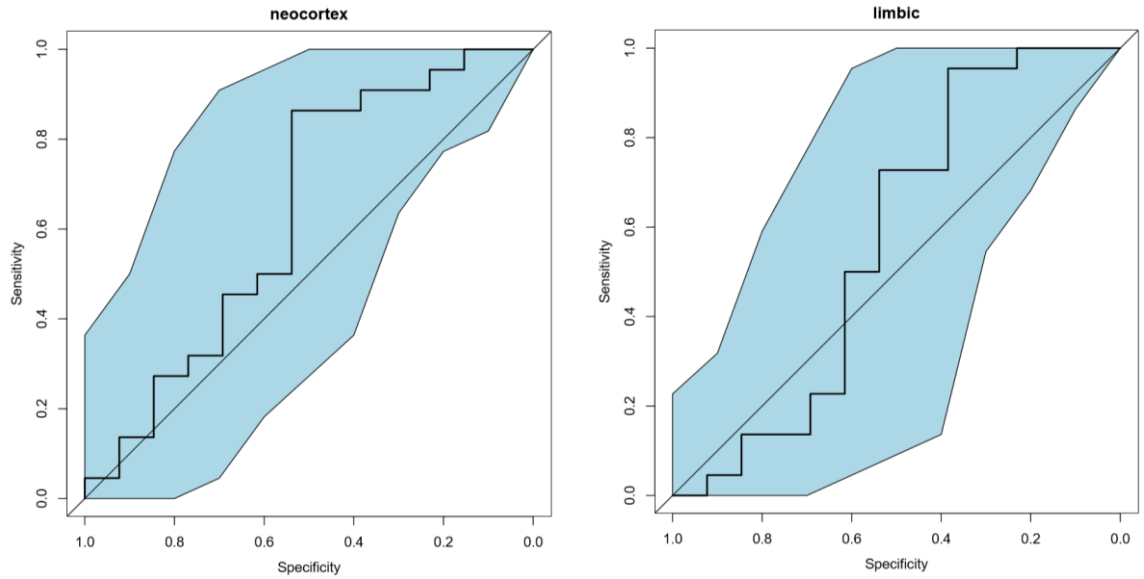
Figure 9. Plots of 5-HT_4R baseline BP_{ND} in neocortex for patients with MDD and healthy controls. A latent variable model was used to test for group differences at a regional level. Mean bars with 95% CI are shown. P-values are adjusted for 3 comparisons. **A.** Healthy controls (n=91) and binary early categorical response at week 4 (early non-responder (n=14), early responder (n=34)). **B.** Healthy controls (n=91) and binary categorical response at week 8 (non-responder (n=13), remitter (n=22)).

Prediction analysis

The results from the prediction analyses are presented in Table 3 and Figure 10. Regional baseline 5-HT_4R binding did not prove any significant power for discrimination of non-responders from remitters at week 8, or early non-responders from early responders at week 4.

	WEEK 4		WEEK 8	
	AUC	**p-value**	**AUC**	**p-value**
Neocortex	0.66	0.12	0.63	0.20
Neostriatum	0.61	0.24	0.57	0.52
Limbic region	0.60	0.35	0.57	0.54

Table 3. Area under the curve (AUC) for the receiver operating characteristic (ROC) curve at week 4 and 8.



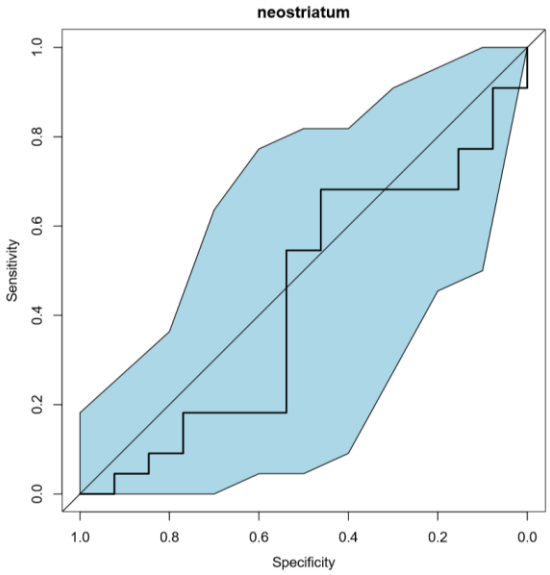


Figure 10. ROC curves for week 8 in the three brain regions. ROC curves (black thick lines) plotted for various true positive rates (sensitivity) against false positive rates (1- specificity) in neocortex, the limbic region and neostriatum. The blue area represents the uncertainty of the curve. The diagonal, thin black line represents the threshold value of 0.5.

Discussion of the longitudinal results

When stratifying patients into the categorical response groups of remitters and non-responders, we found that remitters had 8-10% lower baseline 5-HT_4R binding across all tested regions compared to both healthy controls and non-responders. On the contrary, non-responders did not differ in baseline binding compared to healthy controls. If 5-HT_4R binding is inversely related to cerebral 5-HT levels [72], then the normal binding in non-responders could represent an intact serotonergic system with normal cerebral 5-HT levels. The low 5-HT_4R binding found in remitters could instead signify an alteration of the 5-HT system with increased 5-HT tonus already before treatment, perhaps as an attempt to raise 5-HT levels in order to maintain mentally healthy (as proposed for individuals at familial risk for MDD [71]). Another explanation could be that the lower binding found specifically in remitters characterizes a subtype in MDD with primarily low capacity for 5-HT_4R agonism. As the 5-HT_4R baseline binding differed between remitters and non-responders, it seems unlikely that low 5-HT_4R binding is an overall trait (or state) marker for MDD, but rather applies for a specific subtype, namely those with a favorable response to SSRI treatment. Based on our data, it is not possible to determine whether low 5-HT_4R binding is a state or trait marker in certain depressed individuals. Nevertheless, our

findings could help generate the identification of distinct biological subtypes in MDD; on the one hand non-responders with normal 5-HT_4R binding who might have a different underlying pathology of a "non-serotonergic-related" MDD, and on the other hand remitters with low 5-HT_4R binding who might have a "serotonergic-related" MDD, and accordingly only these patients benefit from serotonergic modulating antidepressants. Interestingly, a recent large meta-analysis (n=17.500) studied the variability in treatment response to antidepressants or placebo and found a higher outcome variability in patients receiving antidepressants compared to placebo. It was suggested that there are moderators systematically associated to antidepressant treatment response, which could be associated with yet unidentified subtypes in MDD [95].

The 5-HT_4R baseline BP_{ND} was a poor predictor for discrimination of clinical response at week 8 and week 4. The lack of predictability is in line with previous PET studies of 5-HT receptors and the SERT, that in general have been unable to convincingly and clinically meaningfully predict antidepressant treatment response [40,42,44,45]

The association between baseline 5-HT_4R binding and treatment response at week 4 was generally stronger than to week 8. We speculate that this could be due to a more initial and direct effect of a serotonergic intervention on the 5-HT_4R, whereas week 4 to 8 might have been "noisier" in terms of either a diminished physiological effect or non-specific factors interfering with the clinical response. On the other hand, escitalopram has been shown to have both an early and sustained effect over time [96]. In general, the treatment response could result from the pharmacotherapy alone, but contribution of non-specific factors such as therapeutic alliance [97] or expectations to the drug-therapy cannot be excluded. A randomized placebo-controlled study might have been able to better account for this, but the aim of this trial was not to study the efficacy of the treatment, but to investigate the 5-HT_4R binding as a biomarker for treatment response in a naturalistic setting following established clinical guidelines.

III. Findings from the rescan assessments

Results and patient characteristics

Forty-three patients were allocated to the re-scan assessment. Two patients (one intermediate responder and one non-responder) were excluded from the analyses because of non-compliance to the medicine (undetectable serum drug-levels at week 8) and 1 patient (remitter) was excluded because of PET-scan data failure, resulting in inclusion of 40 patients in the re-scan analyses. Table 4 describes characteristics of the rescanned patients. There was no mean difference between remitters and non-responders for any of the tested variables except for MDI-score and change in $HAMD_6$ after 8 weeks of treatment (both expected differences). A boxplot of the distribution of changes in $HAMD_6$ score and response categories are shown in Figure 11. One intermediate responder had a higher $HAMD_6$ score at week 8 than at baseline, illustrated as the far-right observation in Figure 11. This patient did not meet the criteria for early non-response at week 4 and, according to the construction of response categories, was therefore not able to categorize as a non-responder at week 8 (even though the depressive symptoms had worsened).

Changes in 5-HT_4R binding after SSRI/SNRI treatment

After 8 weeks of treatment, we found a highly significant decrease in 5-HT_4R BP_{ND} at a global level ($p < 0.0001$) (Figure 12). At a regional level, there was a significant decrease of 9% in the neostriatum (95% CI [-12.8% to -5.0%], p.adj<0.0001), but not in neocortex (-1.4%, 95%CI [-6.2% to 3.6%], p.adj=0.79) or hippocampus (-1.7%, 95%CI [-7.5% to 4.5%], p.adj=0.80). We found no evidence supporting that a change in binding from baseline to re-scan was associated with categorical response (p=0.60), neither to $r\Delta HAMD_6$ (p=0.74) at week 8, in particular also not for neostriatum (p=0.68).

	Non-responder (n=5)	Intermediate responder (n=23)	Remitter (n=12)	Non-responder versus remitter p-value [a]
Females, n (%)	3 (60)	17 (74)	6 (50)	0.73
	Mean (SD)	***Mean (SD)***	***Mean (SD)***	**p-value [b]**
Age	25.3 (4.5)	26.2 (6.1)	29.8 (8.6)	0.46
Years of education	12.0 (0.0)	11.7 (0.6)	11.9 (0.3)	0.48
$HAMD_{17}$ baseline	21 (1.2)	22.4 (3.6)	21.9 (2.8)	0.71
$HAMD_6$ baseline	10.8 (2.6)	12.9 (1.4)	11.8 (1.5)	0.42
%change $HAMD_6$ week 8	-6.4 (8.7)	-54.4 (25.6)	-82 (13.6)	**<0.01**
MDI baseline	37.8 (5.9)	34.3 (6.0)	31.8 (9.1)	0.14
MDI rescan	33.2 (11.4)	20.0 (7.0)	8.9 (4.4)	**<0.01**
Serum drug week 8 (nM)				
- Escitalopram	51 (29)[*]	98 (56)[#]	60.55 (26.5)	0.58
- Duloxetine	140 (142)[**]	92 (NA)[##]	NA	NA
Injected mass (ug/kg)	0.025 (0.037)	0.012 (0.01)	0.0067 (0.002)	0.14
Injected dose (Mbq)	560.4 (41.6)	593.4 (25.4)	593.3 (17.9)	0.14
Reference region binding (cerebellum) (kBq/ml), rescan	11.2 (3.2)	11.0 (2.7)	9.7 (1.7)	0.21

Table 4. Characteristics for rescanned patients at week 8. * n=2, one patient missed the serum drug test at week 8. ** n=2. # n=22, ## n=1. NA: Not Applicable. $HAMD_{17/6}$: Hamilton Depression Raring Scale 17 and 6 items. Group differences between non-responders and remitters was tested using [a] Fisher's exact t-test p value, and [b] Mann Whitney U-test p value.

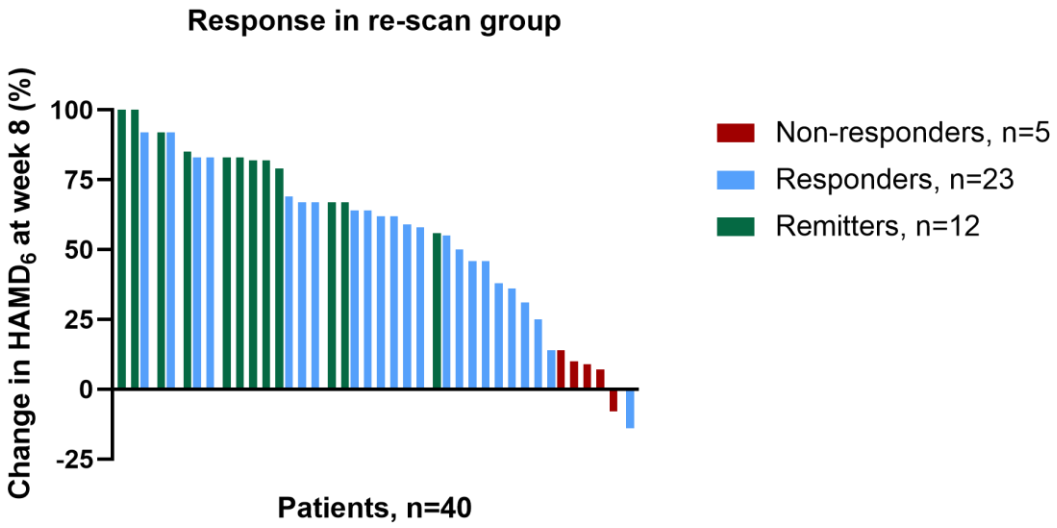


Figure 11. Boxplot of distribution of relative % change in $HAMD_6$ and categorical response groups for rescanned patients. Changes in $HAMD_6$ score are shown such that a positive 100% means full remission, while a negative percentage means worsening of symptoms.

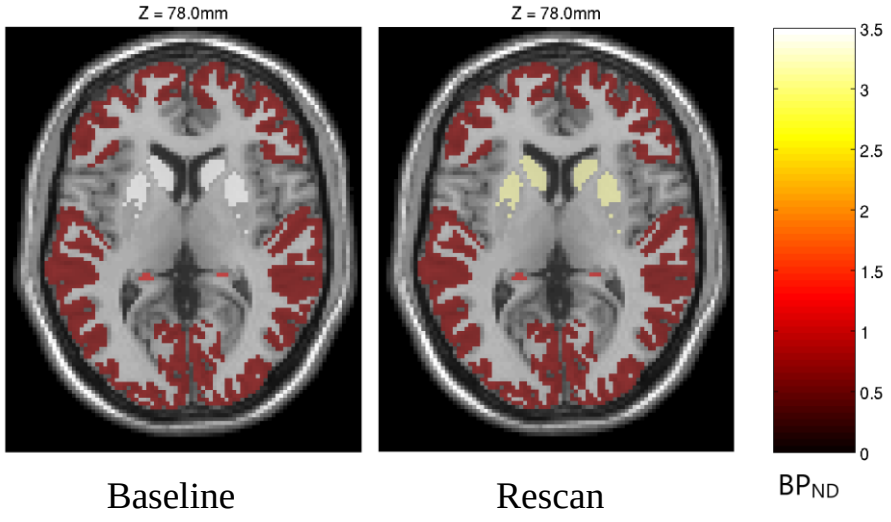


Figure 12. Mean 5-HT_4R BP_{ND} at baseline and rescan for the rescan patient-group (n=40). There was a significant decrease in binding in neostriatum (white area at baseline and yellow area at rescan), but not in neocortex or hippocampus.

Discussion of the findings from the rescan assessment

After 8 weeks of treatment, we found a 9% decrease in 5-HT_4R BP_{ND} in neostriatum, but not in the other brain regions of interest. The decrease was observed regardless of treatment outcome status, which imply that the SSRI/SNRI treatment was pharmacologically effective even if some patients (i.e., the non-responders, maybe with a non-serotonergic MDD) did not profit from the medicine. At baseline, remitters had already a lower 5-HT_4R binding than healthy controls and after successful SSRI treatment, the binding was further reduced, suggesting that even higher 5-HT levels were required to obtain remission in these patients.

Importantly, in a *post hoc* analysis using the same statistical model as Haahr and colleagues [72], we were able to replicate the previous finding of a global decrease in 5-HT_4R binding after 3 weeks of fluoxetine intervention in healthy participants, albeit after 8 weeks in our study (-0.07, 95% CI [-0.11 to -0.037], p=<0.001). Because of the different SSRI intervention protocols between the two studies (3 weeks fluoxetine versus 8 weeks of escitalopram/duloxetine), we do not know how much the decline in neostriatum differ from patients compared to healthy controls, but we find that long-term serotonergic intervention decreases the global 5-HT_4R BP_{ND} also in a depressed cohort.

The regional finding of reduced 5-HT_4R binding after antidepressant treatment was only significant in neostriatum whereas at baseline, global and regional differences between groups were found in all regions of interest. This could mean that 1) a change was detected only in neostriatum because of its high-binding properties, or 2) there is a regional effect on the 5-HT_4R binding after serotonergic exposure that specifically involves neostriatum. Interestingly, a decrease in 5-HT_4R density in neostriatum and hippocampus, but not in the medial frontal cortex, has been found after SSRI intervention in rodents [66], and neostriatum has among the highest density of SERT [56] which is key in the action of SSRI treatment. A few clinical PET-studies have studied 5-HT receptor changes in MDD after chronic SSRI treatment, but with varying results [35,42,43], warranting further studies of regional changes in receptor binding after SSRI intervention.

Methodological considerations

There are some methodological considerations to the study. For example, other studies commonly report characteristics such as age of onset, duration of the current depressive episode and number of lifetime depressive episodes. In this study, we did not specifically collect information about age of onset or duration of the episode, but it was implicated through the exclusion-criteria (<2 years duration of the current episode). A more precise information about these parameters could have contributed to the interpretation of 5-HT_4R binding levels in post-hoc analyses (e.g., association between duration- or total number of depressive episode and 5-HT_4R binding). On the other hand, such data is difficult to quantify because of the risk of recall bias and incorrect information, since patients might not be aware of what and when symptoms define as a depressive episode.

Another methodological consideration is the choice of depression rating scale. A vast amount of studies uses changes in $HAMD_{17}$ to determine treatment response, which also includes items that may be falsely inflated by potential side effects to the drugs, e.g. gastrointestinal or sexual symptoms. This means that one might measure changes in side effects rather than "true" depressive symptoms. The use of the $HAMD_6$ subscale in this study is therefore likely to better monitor changes in core antidepressive symptoms, which generates a more valid treatment outcome [79], while $HAMD_{17}$ can serve as a useful tool to estimate baseline depression severity, and in addition is valuable for comparison with other studies using the full 17 items scale.

A ≥50% change in score from baseline, or a final score ≤7 in $HAMD_{17}$ (usually at week 8 or 12) has commonly been used as cut-off to describe clinical response or remission, respectively [75]. However, this approach is not well suited to distinguish early response. Since patients can respond at various timepoints, the categorization of all remitters as a single group might confound predictors of response. With a construction of categorical response groups that builds on the early response (week 4 in our study), we enable the detection of both early and sustained clinical outcome by generating groups of "excellent" and "poor" responders (i.e., remitters and non-responders).

Patients were allowed anxiolytics and sleeping medicine to reduce side-effects to the medicine and to enhance compliance. We do not know to what extent this might have influenced the treatment response, but since $HAMD_6$ does not include sleep-related items,

and only a few patients (n=4) received anxiolytics and for a short time (< 14 days, and not within 72 h of scanning which was controlled for with urine drug tests), a direct effect on our outcome measure seems unlikely.

The timepoint for when patients were offered to switch from escitalopram to duloxetine was set to week 4 in our trial, which is in line with national guidelines and the commonly used clinical guidelines from the American Psychiatric Association [83]. Previous clinical studies have found that treatment response may be evaluated both before and after 4 weeks. One smaller study found that early non-responders might benefit from switching antidepressant already after 2 weeks [98], while STAR*D reports recommend to continue treatment if just a modest (20%) reduction in symptom score is present after 6 weeks [99]. With the frequent clinical follow-up in this study, patients could reach a maximum dose (20 mg) of escitalopram already after 2 weeks (depending on adverse side effects and clinical response). Therefore, a switch after 4 weeks was considered a reasonable time point for early non-responders to be offered a switch in medicine.

All included patients were evaluated by a general practitioner, went through a baseline clinical interview (including $HAMD_{17}$ and an assessment of whether diagnostic criteria for other disorders, such as bipolar disorders, were met) and were diagnostically verified by a specialist in psychiatry. As such, we believe that the included cohort truly represents patients with MDD (or at least patients who would be treated as depressed within the health care system). However, we cannot rule out the possibility of e.g. bipolar disorder, schizophrenia or other psychiatric disorders/conditions presenting with an initial depressive episode [100,101]. One way to overcome this hypothetical issue would be to follow the patients over time to determine if the MDD diagnosis changed after they had completed the study.

Perspectives

Our findings are intriguing and could generate prospective clinical studies further investigating the involvement of $5\text{-}HT_4R$ in MDD. For example, a study-design with two (or three) consecutive PET-scans both before and after SSRI-treatment, using radiotracers targeting both the $5\text{-}HT_4R$ and e.g. SERT or the $5\text{-}HT_{1A}$ receptor in the same depressed cohort (with the same study protocol) might help further evaluate serotonergic regulations in the depressed brain.

To uncover whether low 5-HT_4R levels is a state or a trait characteristic for certain patients with MDD, it would be interesting to follow healthy individuals genetically disposed for MDD over time, to see if altered 5-HT_4R binding characterizes those with subsequent MDD. In addition, to study the 5-HT_4R binding in not recently medicated patients with MDD in either sustained remission and/or in-between depressive episodes. In the current study, we did not collect sufficiently detailed data about family history of MDD, which otherwise could have helped in studying the association between family-risk and 5-HT_4R binding both in a depressed cohort and as a replication study in the healthy cohort.

Escitalopram has been shown to have a more rapid and sustained antidepressant effect than other SSRIs [96]. If the outcome of our categorical response groups was influenced by a specifically rapid onset of antidepressant effect from escitalopram is uncertain. A replication study using another SSRI would be able to address this matter.

The 5-HT_4R has been emphasized as a possible therapeutic target in MDD. Prucalopride is a high-affinity 5-HT_4R partial agonist, and a recent translational placebo-controlled study showed improvements in memory tasks after only a single dose (1 mg) in healthy participants [62]. In contrast to previous animal studies [58,59,61], antidepressant effects in terms of improvements of emotional processing tasks was not detected [62]. The authors gave potential explanations for this negative finding, for example the short administration time. A next step could be to study patients with MDD to determine the therapeutic effects of 5-HT_4R activation, both in terms of memory and emotional effects as well as in association to the subtype of (non-)serotonergic-related MDD as found in this PET-study. For example, do patients with low baseline 5-HT_4R binding perform better in memory/emotional processing tasks after extended Prucalopride treatment compared to or in addition to SSRI treatment. Also, the subtype with non-serotonergic related MDD would be relevant for studies of treatment outcome in response to non-serotonergic acting antidepressants or alternatively non-pharmacological treatments.

Another intriguing aspect is how the 5-HT_4R binding may be associated with symptoms of anxiety in depressed patients. Anxiety disorders and MDD have considerable comorbidity since up to 50 % of patients with a current depressive episode also meet the criteria for an anxiety disorder [102]. SSRIs are established treatments for both MDD and anxiety disorders, and a shared pathology has been proposed, but like MDD, the pathophysiological understanding of anxiety is

limited. The 5-HT_4R has been implicated also in anxiety since 5-HT_4R agonists yielded anxiolytic effects [59] and protected against stress and anxiety-like behavior in animal models [60]. Future studies could address how and if the 5-HT_4R binding differ in depressed patients with or without comorbid anxiety and compared to healthy controls, and also determine how baseline 5-HT_4R binding correlate with changes in anxiety symptoms and serotonergic treatment response.

Concluding remarks

This is, to our knowledge, the largest clinical PET-study of MDD conducted in a single cohort, and the first clinical *in vivo* evidence for a direct 5-HT_4R involvement in patients with MDD. In summary, we found an average 7-8% lower 5-HT_4R BP_{ND} in neocortex, hippocampus, caudate nucleus and putamen in pre-treatment patients with MDD compared to healthy controls. We were not able to predict treatment outcome at an individual level by using the 5-HT_4R baseline binding as a biomarker. Nevertheless, we found that patients with poor clinical response at week 4 and 8 did not differ in mean 5-HT_4R baseline binding compared to healthy controls, whereas patients with early-response or remission had about 8-10% lower baseline binding than non-responders and healthy controls across all brain regions. We also confirmed a decrease in neostriatum 5-HT_4R binding after serotonergic antidepressant treatment, which in our cohort was independent of clinical response-status at week 8. The findings could be explained by a direct effect of low 5-HT_4R agonism capacity in patients who remit in response to SSRI treatment. In addition, or independently, it could also be a protective mechanism where patients with a subtype of serotonergic related MDD have lower 5-HT_4R binding and increased cerebral 5-HT levels pre-treatment, and where only SSRI treatment realizes levels required to remit. On the other hand, non-responders with normal 5-HT_4R level and a presumed intact serotonergic system do not seem to benefit from serotonergic acting drugs, perhaps because they contain a subtype of non-serotonergic related MDD with a different underlying pathophysiology.

In conclusion, this study provides novel insights and point to PET neuroimaging of the 5-HT_4R as a potentially useful biomarker to aid in the identification of distinct subtypes in MDD, which ultimately may facilitate future strategies for precision medicine.

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Paper I

Predicting treatment outcome in Major Depressive Disorder using serotonin 4 receptor PET brain imaging, functional MRI, cognitive-, EEG-based and peripheral biomarkers: a NeuroPharm open label clinical trial protocol

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Abstract

Background

Between 30–50% of patients with major depressive disorder (MDD) do not respond sufficiently to antidepressant regimens. The conventional pharmacological treatments predominantly target serotonergic brain signaling but better tools to predict treatment response and identify relevant subgroups of MDD are needed to support individualized and mechanistically targeted treatment strategies. The aim of this study is to investigate antidepressant-free patients with MDD using neuroimaging, electrophysiological, molecular, cognitive, and clinical examinations and evaluate their ability to predict clinical response to SSRI treatment as individual or combined predictors.

Methods

We will include 100 untreated patients with moderate to severe depression (>17 on the Hamilton Depression Rating Scale 17) in a non-randomized open clinical trial. We will collect data from serotonin 4 receptor positron emission tomography (PET) brain scans, functional magnetic resonance imaging (fMRI), electroencephalogram (EEG), cognitive tests, psychometry and peripheral biomarkers, before (at baseline), during and after 12 weeks of standard antidepressant treatment. Patients will be treated with escitalopram, and in case of non-response at week 4 or intolerable side effects, offered to switch to a second line treatment with duloxetine. Our primary outcome (treatment response) is assessed using the Hamilton depression rating subscale 6 item scores at week 8, compared to baseline. In a subset of the patients (n=~40), we will re-assess the neurobiological response (using PET, fMRI and EEG) 8 weeks after initiated pharmacological antidepressant treatment, to map neurobiological signatures of treatment responses. Data from matched controls will either be collected or is already available from other cohorts.

Discussion

The extensive investigational program with follow-up in this large cohort of participants provides a unique possibility to (a) uncover potential biomarkers for antidepressant treatment response, (b) apply the findings for future stratification of MDD, (c) advance the understanding of pathophysiological underpinnings of MDD and, (d) uncover how putative biomarkers change in response to 8 weeks of pharmacological antidepressant treatment. Our data can pave the way for a precision medicine approach for optimized treatment of MDD and also provides a resource for future research and data sharing.

The study was registered at clinicaltrials.gov prior to initiation (NCT02869035; 08.16.2016, URL: https://clinicaltrials.gov/ct2/results?cond=&term=NCT02869035&cntry=&state=&city=&dist=)

1 Introduction

Major Depressive Disorder (MDD) is one of the most severe and common brain disorders worldwide with a huge impact on life quality and socioeconomic status [1,2]. It has been linked to serotonergic dysfunction, cognitive disturbances, brain network dysfunction, vulnerability to stress, neuro-inflammation, and gene by environment factors. Still, the understanding of the pathogenesis remains limited. Guidelines for MDD treatment selection are still predominantly based on simple clinical observations about overall MDD severity, and in the case of recurrent depressive episodes, it is also based on personal patient history of treatment responses. Conventional medical treatment is mainly based on intervention of the monoaminergic system in the brain, in particular the serotonin (5-HT) system. Selective serotonin reuptake inhibitors (SSRI) act through blockage and subsequent downregulation of the serotonin transporter (SERT) [3], which presumably induces increased extracellular 5-HT levels. However, robust evidence for a central 5-HT hypofunction in patients with MDD *in vivo* is lacking [4]. Roughly one third of patients suffering from MDD do not respond sufficiently to 5-HT acting drugs [5,6], suggesting a diverse pathophysiology. The diagnostic criteria for MDD may cover a heterogenous collection of various biological entities and consequently, it is unsurprising that a "one size fits all" treatment strategy is suboptimal [7]. Currently, the time from starting to administer a potentially efficacious drug until it can be determined if the clinical response is satisfactory is, at best, 4 – 6 weeks. In clinical practice, the lack of convenient and accurate tools (e.g. quantitative and/or biological) to predict treatment response prolongs the delay from diagnosis to effective treatment and constitutes a major challenge for both clinicians and patients. Therefore, stratification of subtypes and a shift towards precision medicine, e.g., through identification of predictors of treatment response, so-called biomarker(s) that can help optimize treatment choice, is of paramount importance. Candidate biomarkers could be related to neurotransmission, specific neural networks or structural alterations in specific brain regions that can be detected by brain imaging modalities such as positron emission tomography (PET), functional magnetic resonance imaging (fMRI) and electroencephalogram (EEG) or altered biophysiological or cognitive functions [4,8]. It has also been suggested that rather than a single biomarker, an algorithm involving a set of biomarkers may prove useful to subgroup patients and predict their response to certain treatment strategies in MDD [9]. Several biomarkers derived from prior large studies such as

iSPOT-D, EMBARC and CANBIND for prediction of drug response in MDD have been proposed [10–12].

Here, we use multimodalities (PET, fMRI and EEG, cognitive testing, psychometrics and peripheral biomarkers) as part of a deep phenotyping and as a unique feature to our trial, we study modes of action in the brain on a neurotransmitter level. Thus, our trial contributes with novel insights as well as provide a dataset for cross-validation of other identified predictors of psychopharmacological antidepressant treatment response. In a non-randomized, longitudinal, open clinical trial, patients with moderate to severe depression will be treated with SSRI following Danish guidelines. In order to map neurobiological signatures of treatment, we will re-examine a subset of the cohort with neuroimaging and EEG after 8 weeks of SSRI treatment and assess cognitive changes after 12 weeks. This clinical trial is part of a larger research initiative, "NeuroPharm", which addresses pertinent and basic questions regarding human brain disease mechanisms and seeks to predict brain responses to categories of neuro-modulatory interventions as well as treatment efficacy (www.np.nru.dk). We anticipate that this study will critically advance and inform future stratification strategies, further uncover pathophysiological- and treatment mechanisms and, hopefully, guide future precision medicine approaches to optimize treatment strategies for patients suffering from MDD.

Imaging techniques have vastly increased our understanding of the underpinning cerebral mechanisms involved in MDD [13]. Serotonergic dysfunction is considered a central mechanism in depression, and a recent review points at the 5-HT 4 receptor (5-HT_4R) as highly implicated in MDD [14]. For example, 5-HT_4R agonism has shown rapid antidepressant -like behavioral effects in rodents [15], and experimental models suggest that cerebral 5-HT_4R levels are sensitive to central 5-HT modulation in rodents [16,17]. Subsequent clinical studies from our group demonstrated that cerebral 5-HT levels can be indexed in an inverse manner through molecular brain imaging of the 5-HT_4R by using the PET-ligand 11C-SB207145 *in vivo* [18].

We here aim to evaluate 5-HT_4R binding as a candidate predictor of antidepressant response to drugs targeting the 5-HT system in the hitherto largest cohort of MDD patients with PET brain imaging of serotonergic markers. We hypothesize that 1) patients with

MDD differ in cerebral [^{11}C]SB207145 binding at baseline compared to healthy controls; 2) [^{11}C]SB207145 binding at baseline in patients with MDD predicts remission after 8 weeks of pharmacological serotonergic intervention; 3) After 8 weeks of serotonergic intervention, patients with remitter status have a significantly greater reduction in cerebral [^{11}C]SB207145 binding than non-responders. For an overview of primary hypotheses for other modalities, see Appendix 1.

fMRI can be used to assess regional activity and resting state functional networks in MDD. One systematic review found abnormal (negative bias) reactivity in amygdala responsiveness to facial expressions and emotional stimulation in patients with MDD versus healthy controls [19], and pre-treatment low amygdala reactivity has shown to be predictive for antidepressant treatment response [20]. A study with 70 patients with MDD was able to predict treatment recovery with ~80%, by investigating amygdala reactivity to facial emotions and its interaction with history of early life stress [21]. Another study from our group showed that amygdala reactivity was associated with brain 5-HT$_4$R binding and hence putatively extra synaptic 5-HT levels in healthy individuals. This established a plausible connection between 5-HT levels and amygdala activation, both involved in emotional cognitive processes [22]. This exemplifies how a multimodal PET and fMRI strategy can highlight molecular mechanisms mediating drug effects on brain function [23]. Resting state fMRI (rs-fMRI) measures fluctuations in fMRI signal during the absence of an explicit task and is widely used to assess distributed intrinsic networks such as the "default mode network" [24]. Alterations in rs-fMRI connectivity have been described in MDD [25] and a recent study suggested that rs-fMRI can define subtypes of MDD and predict antidepressant treatment response [26], but this has been contested by others [27]. Although promising, brain imaging studies have in general been inconclusive and with small sample sizes [9,28]. In the current trial, we will use task-based and rs-fMRI in a large cohort of patients with MDD and investigate the association between 5-HT$_4$R levels (as a proxy for brain serotonin levels) and the clinical outcome of SSRI treatment.

EEG, a monitoring technique for direct ongoing neural activity, has been reported to be associated with treatment response in MDD (see, e.g. review [29]). Prior studies have found that treatment responders have higher cortical alpha activity [30] and higher theta activity at rostral anterior cingulate cortex compared to treatment non-responders [31,32]. Of note, these

biomarkers were derived from the resting EEG data, which is relatively easy to implement in the clinic. Furthermore, earlier evidence from event-related-potential (ERP) studies have suggested that ERP biomarkers such as auditory P300 (a positive waveform around 300 ms after stimulus onset) and loudness-dependence of auditory evoked potentials (LDAEP) can be predict drug treatment response [33,34], and are linked to the serotonergic transmitter system [35]. In the current trial, the predictive values of pretreatment EEG/ERP biomarkers will be examined.

Disturbances in cognitive processes including memory, attention, and executive functions are commonly reported in MDD [36] and contribute to psychosocial impairment and workforce disability [37]. In addition, affective bias in information processing (i.e., favoring negative information over positive information at different levels of information processing) has been proposed as a central mechanism in the development and maintenance of depressive symptoms [38] which is also predictive of later treatment response to antidepressant drugs [39]. Notably, cognitive disturbances do not always resolve with the remission of a depressive episode, suggesting a dissociation between core mood and cognitive symptoms in MDD [40]. Combined with the low cost and relative ease of testing in a clinical setting, this distinguishes cognitive disturbances as a promising marker for stratification of depression subtypes as well as an important target for antidepressant treatment. In the present study, we therefore aim to map a broad range of cognitive disturbances in MDD, including both cold (non-emotional) and hot (emotional) cognitive processes, and explore whether they may be used to characterize clinically relevant subgroups in MDD. Based on earlier observations in healthy individuals, we expect memory performance to map onto hippocampal 5-HT_4R availability [41] and possibly affective bias in verbal memory in MDD [42].

Evidence of inflammation-associated MDD has emerged over the years [43]. Patients with MDD show elevated levels of inflammatory markers in peripheral blood [44] which may affect treatment response such that higher levels are associated with worse response [45]. It has also been suggested that patients with MDD have higher levels of activated microglia, as illuminated with PET [46]. Proinflammatory cytokines may influence the 5-HT homeostasis in the brain by acutely upregulate SERT through intercellular pathways (i.e. linked to p38 mitogen-activated protein kinase) and presumably thereby reduce synaptic 5-

HT levels [47]. Interestingly, cognitive dysfunction, a prevalent symptom in depression, also appear to be linked to an inflammatory response [48]. We here aim to determine if higher levels of systemic inflammatory markers are associated with 5-HT_4R brain binding, depression status at baseline and clinical treatment response.

Another area of interest is the association between MDD and signatures of early aging. There is an increased mortality and prevalence of age-related diseases in recurrent depression [49,50]. Oxidative stress on nucleic acids is a general element of aging and has been suggested to be an underlying biological mechanism of the accelerated aging observed in depression [51]. Previous research from our group has found evidence for such a link, both in studies of psychological/biological stress and oxidative stress in patients and in rodent models of depression [52–54]. Earlier findings indicate alterations in levels of oxidative stress during antidepressant treatment and it is hypothesized that treatment response is related to a transient increase in oxidative stress levels, perhaps due to neurotrophic processes and/or peripheral changes in energy metabolism [55–57]. Urinary 8-oxodG and 8-oxoGuo are sensitive and specific markers for systemic DNA/RNA damage from oxidation [58]. We here aim to investigate urinary 8-oxodG and 8-oxoGuo as a predictive biomarker for antidepressant treatment response, its association with changes in psychopathology, structural and functional brain changes, and markers of psychological and biological stress. Additionally, we will investigate whether hormonal (estradiol, testosterone, progesterone and follicle-stimulating hormone (in females)) and metabolic status can predict antidepressant treatment response and explore whether these associations are related to genetic make-up (specified below), psychopathology and the occurrence of early life stress using self-reported childhood adverse events and parental bonding quality questionnaires, which also may interact with the 5-HT system [59,60].

Sexual dysfunction (e.g. low sexual desire, arousal difficulties and anorgasmia) is a prominent feature of Major Depressive Disorder (MDD), which often leads to a decline in quality of life [61,62]. Lack of interest to what is usually pleasurable, i.e. anhedonia, is a core symptom in MDD and may also be reflected in reduced sexual desire/interest. Sexual dysfunction, in particular anorgasmia and sexual arousal difficulties may further be linked to serotonergic dysfunctions [63] as seen in MDD. As a complicating factor, impaired sexual health related to MDD may worsen with antidepressant treatments targeting the 5-HT

system. For example, in a group of 704 patients with MDD treated with an antidepressant drug (SSRI) or serotonin-norepinephrine reuptake inhibitor, about half of them developed or experienced worsening in their decreased sexual desire as a side-effect, which was also associated with reduced quality of life, lower self-esteem and adverse effects on mood and partner relations [62]. We currently do not know which patient characteristics predict sexual dysfunction in response to SSRI treatment. However, differences in individual serotonergic brain architecture and/or serotonergic response to antidepressant treatment (e.g. SSRI) may play a role. In this study, we aim to map the frequency and predictors of SSRI induced sexual dysfunction and determine if serotonergic tonus (measured by 5-HT_4R PET binding) pre-treatment, or changes in response to SRRI treatment, is associated with sexual desire and/or development of SSRI-induced sexual dysfunction in MDD.

Previous findings from our group have repeatedly demonstrated a coupling between key features of the 5-HT system and hypothalamus- pituitary- adrenal axis (HPA-axis), which regulates the release of the stress-hormone cortisol [64]. Such HPA-axis dynamics can be measured by the cortisol awakening response. Our results support that both serotonin transporter availability [65], and serotonergic tone or direct capacity for 5-HT_4R agonism [64] support a healthy cortisol response to HPA-axis stimuli. A well-functioning and dynamic HPA-axis is critical for coping with everyday life stressors, and HPA-axis dysregulation is a prominent feature of major depressive disorder. Although heightened CAR is associated with relapse of depressive episodes in patients with a history of depression [66], in the more advanced depressed stages, i.e., chronic depression, HPA-axis dynamics are blunted as opposed to recent-onset depression [67]. Notably, normalization of the HPA-axis in response to SSRI treatment appears to protect against relapse [68]. Thus, the SSRI treatment response is likely to depend on restoring HPA-axis dynamics at least in a subgroup of MDD patients. In this trial, we will assess CAR in patients with MDD, the effect of SSRIs on CAR, investigate its association with baseline 5-HT_4R distribution, as well as evaluate CAR as a predictor of antidepressant treatment outcome.

Materials and methods

Figure 1 shows a flowchart of the scheduled data collection over the 12 weeks of pharmacological drug treatment of patients with MDD. Healthy controls (HC) will be recruited as specified below. Patients will be examined before (at baseline; week 0) and after 1, 2, 4, 8 and 12 weeks of SSRI treatment has been initiated. Depression-severity will be monitored by the Hamilton Depression Rating scale 17 items ($HAMD_{17}$) and its subscale of 6 items ($HAMD_6$) [69]. A subset of patients will be offered re-examination with PET, fMRI and EEG after 8 weeks of treatment, to assess changes from baseline and its association to treatment response. Patients from the whole spectrum of treatment responses (from poor to excellent) will be invited in a continuous fashion for this part of the study until allotted re-examinations are completed. All patients will also repeat cognitive testing at week 12.

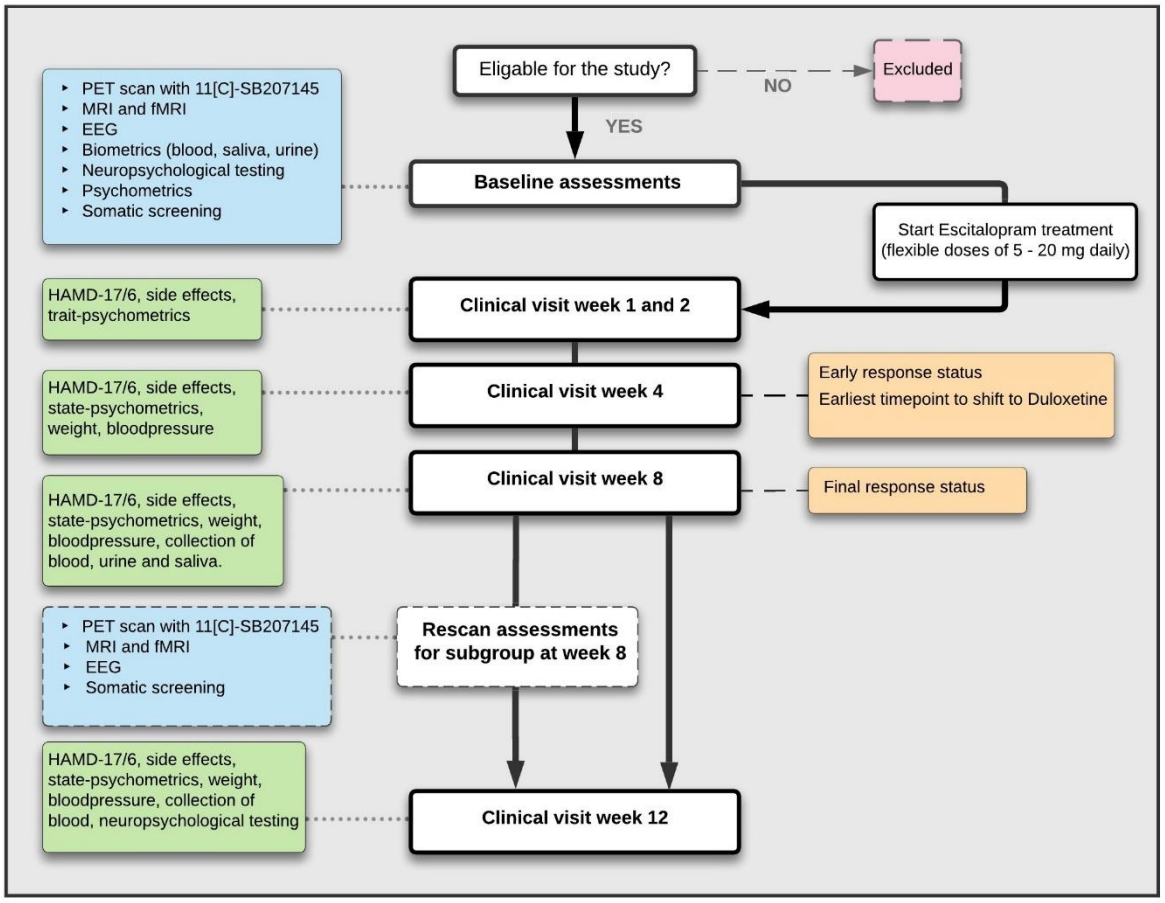

Figure 1. Flowchart of study trial assessments for patients with MDD.

The power analysis in preparation of the study was primarily anchored to the PET modality. We estimated that we needed to include 100 patients to reach a statistical power of 80 % to detect an association between treatment response (binary classification, i.e. remitters vs non-responders, see response-definition below) and baseline 5-HT_4R non-displaceable binding potential (BP_{ND}). These calculations were based on an expected 20 % maximum drop-out, ~ 50% remission rate after 8 weeks of treatment [5,6] and an expected difference of 8 % in 5-HT_4R binding between remitters and non-responders, corresponding to the previously found effect sizes on 5-HT_4R change in BP_{ND} after fluoxetine treatment [18]. Calculations were further based on an average BP_{ND} of 0.71 and a standard deviation of 0.073 [18,70]. With a rescan subgroup of approximately 40 patients, and based on an evaluation of the average changes in $HAMD_6$ seen in the first 31 patients who were included, and a Gaussian distribution of change in BP_{ND} with an SD of 0.08 (log scale), we had an expected power of 80% to identify a significant association between longitudinal changes in BP_{ND} and changes in $HAMD_6$ (i.e., secondary clinical outcome, continuous scale).

1. Participants

Patients are recruited from a central referral center within the mental health services in the Capital region of Denmark or directly referred from one of five general practitioners in collaborations with the study group (see figure 2 (CONSORT) for details). Data from healthy controls for the purpose of baseline comparisons to patients with MDD are available from a pre-existing database on site [71]. The healthy control reference population will be supplemented with newly recruited healthy controls from a local volunteer database (www.nru.dk), as necessary.

1.1 Inclusion criteria for patients

Patients between 18 – 65 years of age with a moderate to severe, single or recurrent episode of MDD consistent with the Diagnostic and Statistical Manual of Mental Disorders -5 (DSM-5) and International Statistical Classification of Diseases and Related Health Problems -10 (ICD-10) criteria will be recruited by a trained clinician. Inclusion requires a total score of >17 on $HAMD_{17}$ at baseline and the diagnose is confirmed by using the

diagnostic tool Mini International Neuropsychiatric Interview [72]. In addition, all patients are diagnostically verified by a specialist in psychiatry before final inclusion.

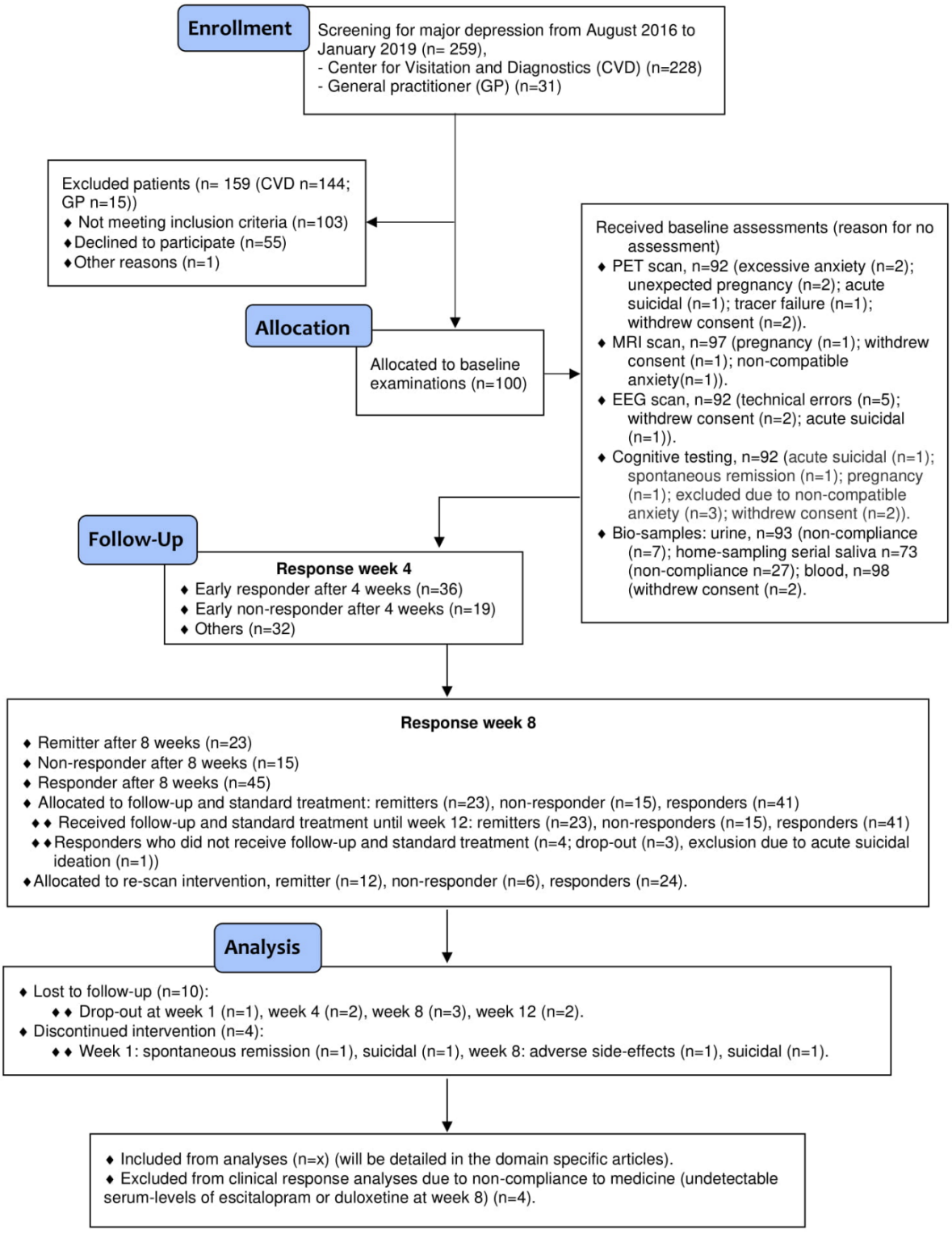

Figure 2. Flow diagram (CONSORT) of the NeuroPharm trial

1.2 Exclusion criteria for patients

Patients with a duration of their present depressive episode exceeding two years are not included. No more than one antidepressant treatment attempt in the current episode prior to inclusion is allowed and only patients with no antidepressant medication within the last two months are eligible. Patients with known contraindications or previous non-response to an SSRI drug after an adequate trial as well as a prior or present history of other primary axis I psychiatric disorders are not included, i.e., MDD must be the primary diagnosis. Other exclusion criteria are: severe somatic illness; substance or alcohol use disorder; insufficient language skills to undergo clinical assessments; acute suicidal ideation or psychosis; patients who are deemed by a psychiatrist to require other forms of antidepressant treatments; pregnancy or breast feeding; use of any CNS drug that cannot be washed out prior to participation (e.g. metoclopramide, ondansetron, serotonergic migraine medicine, clonidine); medical conditions interfering with measurements, contraindications for PET and/or MRI scans; exposure to radioactivity > 10 mSv within the last year; severe sensory or intellectual impediments interfering with comprehension of procedures or assessments and lastly any history of brain injury (i.e. loss of consciousness and amnesia or symptoms of concussion disorder).

1.3 Inclusion and exclusion criteria for healthy controls

Enrolled HC will be sought to match the patient population by gender and age distribution. All HC will be screened for MDD using a self-reporting questionnaire (major depression inventory) [73]. The HC meet the same inclusion and exclusion criteria as required for patients apart from psychiatry related issues (e.g., no current or history of mental illness or unstable somatic condition).

2. Treatment and investigation program

2.1 Baseline assessments before treatment

Each patient will receive a basic physical screening including somatic status, routine blood samples, electrocardiogram including QTc interval and collection of toxicology urine tests (The Rapid ResponseTM Multi-Drug Test Panel (Urine)) for detection of drug abuse

within the last month. Women are screened for pregnancy through self-reported use of contraceptives and a pregnancy urinary test if relevant. All study-participants will undergo baseline assessments of brain imaging with 11C-SB207145 PET and fMRI; EEG-examination; cognitive testing, collection of questionnaires and biological material (venous blood, urine and saliva) as specified below. All HC will receive corresponding baseline assessments.

2.2.1 Clinical procedure after treatment initiation

After completion of baseline examinations, patients will receive flexible doses of the SSRI drug escitalopram, initially 5 mg for 3–5 days depending on side effects (e.g. nausea), followed by 10 mg daily until their first follow-up visit and further adjusted individually to a maximum dose of 20 mg. Escitalopram was chosen as it binds with high selectivity to the 5-HT transporter and has minimal affinity for other receptors [74]. Patients are allowed short-time treatment with cyclopyrrolone (a nonbenzodiazepine hypnotic agent) or oxazepam (a benzodiazepine) to reduce anxiety and sleep disturbances which may be prominent in the initial treatment phase and have shown not to influence treatment outcome [75], but all are requested to avoid use 3 days prior to brain scans. Clinical follow-up sessions with a study physician or trained research assistant are

scheduled in an out-patient clinical setting after 1, 2, 4, 8 and 12 weeks of treatment to evaluate treatment response and side effects. Visits can deviate a maximum of one week from the original time scheduled. No cognitive behavioral therapy or other psychotherapy program is provided during clinical visits. No treatment (pharmacological or psychotherapeutically) other than the medical monotherapy provided in this study is allowed elsewhere during the trial. At week 4, early non-responders (see definition below) or patients with unacceptable side effects are offered to switch to a standard second line antidepressant treatment; duloxetine (individually adjusted doses of 30–120 mg per day), which is a serotonin-norepinephrine reuptake inhibitor. Duloxetine was chosen according to clinical guidelines for second line antidepressant treatments and due to its negligible affinity to the 5-HT_4R [76]. The week 4 timepoint is in line with national guidelines in Denmark for switching to a second-line antidepressant treatment (4-6 weeks). Since our

cohort receives frequent clinical follow-up sessions, patients can reach max dose of escitalopram (20 mg daily) already after 2 weeks. As such, switching after 4 weeks is considered appropriate for early non-responders in this trial set-up. All antidepressant medicine will be provided for free to improve compliance. Compliance will be assessed by serum escitalopram/duloxetine blood tests after eight weeks of treatment as well as tablet count at each follow-up. At each visit, depressive symptoms are rated using the $HAMD_{17}$ and the $HAMD_6$ subscale. $HAMD_6$ captures core symptoms of depression more directly (and disregards sleeping quality), and has been found to be sensitive to antidepressant treatment response [69]. Potential side effects due to intervention will be monitored at each visit using the "Udvalg for Kliniske Undersøgelser" scale [77]. To ensure agreement and allow alignment of ratings, $HAMD_{17}$/$HAMD_6$ co-ratings between all the clinical investigators will be regularly performed during data collection. A maximum of 20 % deviation from the "gold-standard" chief psychiatrist is allowed, or else a new satisfactory co-rating is needed before independent rating of study participants.

2.2.2 Clinical response status

Primary clinical outcome measure

The primary outcome measure is categorical and built to capture patients with an early as well as sustained, either excellent or poor response to treatment. Patients are classified as either "remitters", "non-responders" or "intermediate responders" after 8 weeks of treatment (Figure 3). These categories are based on percentage changes of depressive symptoms from baseline, as measured by $HAMD_6$. Remitters must have ≥ 50 % reduction in $HAMD_6$ at 4 weeks (early responders) and a $HAMD_6$ score < 5 after 8 weeks of treatment. Non-responders have < 25% reduction in $HAMD_6$ after 4 weeks (early non-responder) and < 50% reduction in $HAMD_6$ after 8 weeks of treatment. Patients who do not meet the criteria above, are defined as "intermediate responders" at week 8. The primary predictor analyses are directed to predict treatment response in a binary fashion (either remitter or non-responder).

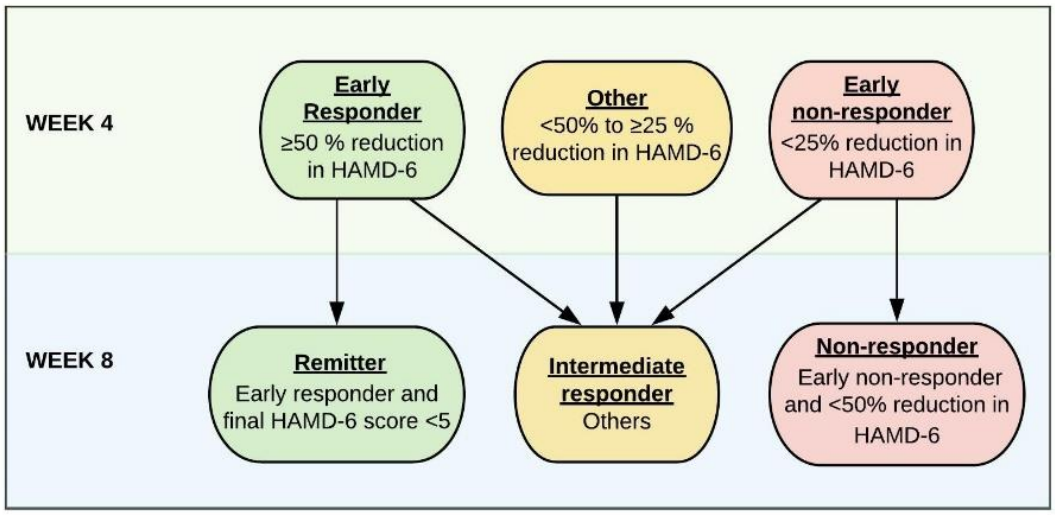

Figure 3. Response categorization for patients with MDD after 4 weeks and 8 weeks of antidepressant treatment based on changes in $HAMD_6$ score.

Secondary clinical outcome measure

As a secondary outcome, we use a continuous response measure, i.e. $HAMD_6$ changes from baseline at week 8 divided by $HAMD_6$ at baseline, to allow analyses of the association between antidepressant treatment response and baseline characteristics or treatment-induced changes in the neurobiological modalities of interest.

3. Examination modalities

3.1.1 PET imaging and quantification of 5-HT_4R brain binding

PET scans are conducted using a high-resolution research tomography Siemens PET scanner (CTI/Siemens, Knoxville, TN, USA) (256 × 256 × 207 voxels; 1.22 × 1.22 × 1.22 mm). Participants are positioned uniformly in spine position and a specialized head holder is applied to reduce head motion during the scan. All participants undergo a 6 min transmission scan and are given an intravenous bolus of approximately 600 MBq of the PET tracer ligand [^{11}C]SB207145. The bolus is administered over 20 seconds followed by a 120-minute dynamic PET data acquisition. The radioligand is synthesized immediately prior to injection as described elsewhere [78].

3.1.2 Preprocessing and PET quantification

The 120 minutes dynamic PET acquisitions are reconstructed into 38 time frames (6x5 s, 10x15 s, 4x30 s, 5x2 min, 5x5 min, and 8x10 min s) using a 3D-OSEM PSF algorithm (16 subsets and 10 iterations) [79] and TXTV-based attenuation correction [80]. For motion correction, the AIR 5.2.5 software will be used, aligning each PET frame to the initial five-minutes frame. Structural 3-Tesla MRI scans will be used for co-registration of the PET images with SPM8 software. Automatic delineation will be carried out in a user-independent manner in PVElab software [81] and mean tissue activity curves for grey matter volumes will be extracted for kinetic modeling. No partial volume correction will be performed because of the high resolution of the scanner. Regions of interest (ROI) have been chosen due to their known relevance in mood disorders and abundance of 5-HT_4R density [82]. The selected ROIs for the primary analyses are neocortex, putamen, caudate nucleus and hippocampus. Co-registration and correct ROI placement for all subjects will be inspected in three planes by a trained investigator. PMOD version 3.0 (PMOD, Zurich, Switzerland) will be used for kinetic modeling and quantification of the 5-HT_4R binding is performed using non-displaceable binding potential (BP_{ND}) as the final outcome measure. The simplified reference tissue model will be used with cerebellum (excluding vermis) as reference region which previously has been validated in humans [83]. BP_{ND} is defined as:

$$\boldsymbol{BP_{ND}} = \frac{\boldsymbol{f_{ND}} \times \textbf{Bavail}}{\boldsymbol{K_D}}$$

where f_{ND} is the tissue free fraction of non-protein bound ^{11}C-SB207145, B_{avail} is the concentration of available 5-HT_4R and K_D is the dissociation constant for the tracer at equilibrium. Thus, BP_{ND} is proportional to the density of 5-HT_4R.

3.2 MRI and fMRI imaging

All participants are screened for MR-compatibility and thoroughly instructed how to perform the fMRI paradigms by a trained study assistant who uses standardized instructions. All MRI scans for patients will be acquired using the same Siemens 3-Tesla Prisma scanner with a 64-channel head coil. High-resolution structural T1- and T2-

weighted MR images will be acquired. Blood oxygenation level dependent fMRI scans will be obtained during a commonly used emotional faces paradigm [84,85], reward-related guessing paradigm [86,87] and a 10-min rs-fMRI scan. During the rs-fMRI scan, participants are asked to close their eyes, let their mind wander and to not fall asleep. All structural scans of patients will be screened for pathological abnormalities by a medical specialist in radiology.

3.3 EEG

EEG data is recorded using a 256-channel HydroCel Sensor Net system (EGI, Inc., Eugene, OR) at 1000 Hz with 0.1-100 Hz analog filtering where vertex electrode serve as the reference. Impedances across all electrodes are kept below 50 kΩ. EEG/ERP recording at baseline included: resting EEG (with eyes closed and open), two-tone auditory oddball and the LDAEP tasks. The same EEG/ERP recording will be re-tested in a subgroup of patients after 8 weeks of treatment.

3.3.1 Resting EEG

Resting EEG is recorded during four 3-min periods with a counterbalanced order of OCOC (O for eyes open, C for eyes closed) or COCO between subjects. Participants are instructed to remain still and relax, avoid eye-blinks and movements and to relax chin muscles during recording. Absolute and relative powers are computed using the following frequency bands: δ (1–4 Hz), θ (4–8 Hz), α (8–12 Hz) and beta (8–30 Hz). In addition, alpha peak frequency (APF) is identified by the frequency at maximal absolute power from the spectral range of 7–13 Hz. Frontal alpha asymmetry will be calculated using alpha power with the formula of (F4 – F3)/ (F4 + F3) (Arns et al., 2015). Furthermore, theta activity will be extracted from anterior cingulate cortex with exact low-resolution electromagnetic tomography (eLoreta).

3.3.2 Task elicited ERPs

The two-tone auditory oddball paradigm consists of two acoustic stimuli with different frequencies. Participants are presented with a series of standard tones (500 Hz) and deviant tones (1000 Hz) binaurally through inserted earphones (Etymotic Research Inc., ER 3C).

They are instructed to press a button when the deviant tones are presented while ignoring the standard tones. ERP components such as N1 and P3 will be computed, both peak latency and amplitude (baseline to peak) will be extracted by the averaged trials. Participants are presented with five acoustic stimuli with different intensities (60, 70, 80, 90, and 100 dB SPL) in the same frequency of 1000 Hz. No response is needed. The primary outcome is the slopes of peak-to-peak N1/P2 amplitudes extracted from the average trials at each intensity. A more comprehensive description of the EEG data will be presented in the subsequent reports.

3.4 Cognitive testing

All participants undergo cognitive testing using selected tasks from the novel test battery EMOTICOM, assessing affective and social cognition including emotional face recognition, emotional threshold detection, theory of mind, and moral emotions [88]. In addition, affective memory [89], working memory, reaction time and IQ will also be assessed. Testing is planned and conducted by trained neuropsychologists prior to start of drug intervention and again after 12 weeks of treatment.

3.5 Psychometrics

Apart from clinical visits including $HAMD_{17/6}$ ratings, patients will apply self-monitoring during the study period and fill out Danish versions of online questionnaires throughout the study. All questionnaires will be imported directly to an internal database through LimeSurvey, a free and open source software. Before EEG scans and cognitive testing, all participants will report their current mood state using an in-house Likert-scale. An adjusted Likert-scale will be filled out after each MR-scan. During visits at week 4, week 8 and week 12, patients are also asked to fill out a comprehensive set of self-rating state questionnaires (see table 1 for a full overview). Healthy controls will be asked to fill out selected state questionnaires as part of their baseline assessments.

3.6 Biomaterials

3.6.1 Blood

At baseline, all participants will be screened for basic somatic status to exclude somatic conditions with possible influence on depressive symptoms. Blood samples will be collected throughout the study (see table 2 for a full overview) for determination of inflammatory status (high sensitivity C-reactive protein, tumor necrosis factor-α, Interleukin-6, -18 and -10) [90–93]; epigenetic variations (SERT, FKBP Prolyl Isomerase 5, Catechol-O-Methyltransferase (COMT), monoamine oxidase-A, glucocorticoid-, estrogen-, oxytocin receptor and oxytocin gene-methylation); extraction of DNA for genotypes of relevance (rs41271330 [94], serotonin-transporter-linked polymorphic region (5-HTTLPR) [70], COMT, Brain-Derived Neurotrophic Factor val66met) and ABCB1, FZD7 and WNT2B (that presumably influence responsiveness to pharmacological antidepressant treatment [95]). At week 8, serum samples of the antidepressant drug (i.e. escitalopram or duloxetine) are collected as trough concentrations in steady state, with primary purpose of monitoring compliance. The samples will be stored at -20 °C (or -80 °C for plasma EDTA samples) until analyzation in batches at completion of the trial. Quantification of escitalopram and duloxetine in serum will be performed at the Laboratory of the Danish Epilepsy Centre, Filadelfia, using a routine UPLC-MS/MS method developed in-house. Standard operating procedure instructions have been established before trial initiation and will be followed during the assessment of all biomaterial.

3.6.2 Saliva

Saliva will be collected to determine the total cortisol output across one day as well as dynamics of the HPA-axis, as indexed by CAR. Serial saliva samples will be sampled at home and collected at baseline and at week 8 (see table 2). Those visits will be placed as close to the PET-scan day or week 8 visit as possible, and patients are instructed to take samples immediately after awakening and again after 15, 30, 45 and 60 minutes, at 12 pm, 6 pm and 11 pm. Participants are also instructed to collect saliva samples preferably during weekdays, not perform strenuous exercise < 2 hours and not to have any oral intake or brush their teeth < 1 hour prior to sampling. Cohen's Perceived Stress Scale and basic information about sleep and food intake will be filled out in conjunction with the home-sampling. All participants receive careful training in saliva collection, instructions of home-sampling procedures; cold storage of samples and fast delivery either by mail or

personal delivery to the laboratory facility for preparation. When received, salivary test-tubes are centrifuged and stored at -80 °C until later single-batch analysis.

3.6.3 Urine

Spot-urine samples will be collected at baseline and week 8 visits for patients (see table 2) in 2 ml Eppendorf tubes and will be stored at -20 °C for later single-batch analysis. Apart from pregnancy and drug-screening (see section 2.1), all urine samples will be analyzed for 8-oxodG and 8-oxoGuo markers for systemic DNA/RNA damage with ultra-performance liquid chromatography with tandem mass spectrometry and normalized to urinary creatinine [96].

4. Statistical analyses

4.1 Evaluating associations between baseline measures, changes from baseline measures, and clinical outcomes

Baseline data from each modality of interest, i.e. PET, EEG, fMRI, MRI, cognitive measures, peripheral molecular markers, and clinical/demographic patient profiles, will be available for evaluating associations with the clinical outcomes for the entire group (n=100 included). Changes from baseline data will be available for the subgroup (around n=40 invited), who will be re-examined with brain scans and EEG for evaluating an association between changed measures and clinical outcomes. Primary analyses will test mean differences in baseline measures of the biomarkers from each modality between healthy controls and patients as well as response groups (remitter vs. non-responder at week 8, i.e. primary clinical outcome) using multiple linear regressions. This analysis focuses on the two extreme outcome groups. Secondary analyses will test the association between baseline measures of the biomarkers from each modality and antidepressant treatment response on a continuous scale, i.e. relative change in $HAMD_6$, using linear multiple regression. This analysis incorporates the full spectrum of clinical outcomes. Similar analyses will be performed to study the association between the change from baseline measures of the biomarkers and the clinical outcomes. Regression models will be adjusted for age and sex, as well as modality-specific relevant covariates. For instance, 5-HTTLPR

status is predictive of 5-HT_4R binding [70] and will be adjusted for in the analyses concerning 5-HT_4R. When relevant, interactions will be evaluated, e.g., we will test if the association between the clinical outcome and 5-HT_4R is moderated by inflammatory status. Diagnostic regression tools will be used to assess model's assumptions (e.g. linearity of the effects, normality assumption for residuals). When violated, corrective procedures will be used (e.g., splines and bootstrap resampling) [97]. As appropriate, adjustments for multiple comparisons will be performed within each modality. In the analysis of the PET data, we will instead use a Latent Variable Model relating the 5-HT_4R binding in several brain regions (neocortex, caudate nucleus, putamen and hippocampus) to treatment outcome via a latent variable [98]. This allows us to assess the association between 5-HT_4R binding and clinical outcome with a single test. Patients are considered with un-verified compliance if they have taken less than 2/3 of their antidepressant medicine, missed their week 8 visit, or have undetectable serum drug levels at week 8 (i.e. <10 nM for escitalopram and < 15 nM for duloxetine). Patients with un-verified compliance will not be included in primary longitudinal analyses of treatment response. Missing data will therefore be handled using complete case analysis which in regression models is valid when the probability of dropping out of the study is, conditional on the covariates, independent of the outcome. If any participants were to be excluded during the study because of their clinical outcome, a sensitivity analysis will be performed.

4.2 Evaluation of the predictive value of the biomarkers within modality

Logistic regression models for the primary clinical outcome will be used to obtain the probability of each patient to be a remitter (vs. a non-responder) based on its clinical data and the value of a modality-specific biomarker. Given a threshold (e.g. 0.5), patients with an estimated probability greater than the threshold will be predicted to be remitters, otherwise to be non-responders. The receiver operating characteristic (ROC) curve will be used to assess the compromise between sensitivity and specificity of this classification across thresholds. Since a 33% remission rate is expected in treatment regimen comparable to ours [5], we will focus on the ROC curve with high-specificity. The AUC (area under the curve) of the relevant part of the ROC curve will be reported as a summary of the predictive performance of each biomarker. The classification performance (accuracy, positive predictive value, negative predictive value) at the threshold optimizing the sum specificity and specificity will also be reported. To limit optimistic biases, these measures

will be estimated using 5-fold cross-validation [99]. A permutation procedure will be used to obtain the null distribution of the predictive performance, against which the observed performance will be compared. Additional classification schemes may be considered (e.g., responder status as defined by ≥ 50% reduction in $HAMD_6$ at week 8), with appropriate adjustment for inflated type-I error, to facilitate comparison of the current data with other relevant clinical trials. The predictor performance will be evaluated in a modality specific fashion and at a next stage, combined predictors will be evaluated.

4.3 Predictive value of the biomarkers across modalities

Two strategies will be considered to optimize prediction of treatment response using biomarkers measured at baseline across modalities. In the first strategy, we will combine the specific biomarker-candidates across all modalities (as predefined in Appendix 1), which will generate around 50 candidate biomarkers. A dimension reduction step will be used to define a small number of predictors (roughly 5-10) that will be used in a logistic regression model. The second strategy will use an algorithm to (i) identify, in a data-driven way, biomarkers with a predictive value among all the existing biomarkers (roughly 5000-10000) and (ii) predict treatment response based on the identified subset of informative biomarkers. We will investigate the use of machine learning methods (e.g. random forest, neural networks) as well as ensemble methods (e.g. Super Learner [100]). The assessment of the predictive performance of these strategies will be carried out as described in the previous section.

5. Ethics approval and consent to participate

The study protocol complies with the Declaration of Helsinki II and data collected during the trial will be monitored throughout the study period (for every 10th patient included) by an independent Good Clinical Practice unit in the capital region of Denmark (www.gcp-enhed.dk/en). The study has been approved by the Committees on Health Research Ethics in the Capital Region of Denmark (reference number: H-15017713), the Danish Data Protection Agency (04711/RH-2016-163) and Danish Medicines Agency (protocol number: NeuroPharm-NP1, EudraCT-number 2016-001626-34). All potential participants will receive oral and written information about the study by the enrolling clinician, and all

enrolled participants will provide written informed consent prior to inclusion. Adverse events have been scheduled to be reported annually to the Danish Medicines Agency. The study was registered at clinicaltrials.gov prior to initiation (NCT02869035), date: 08.16.2016.

6. Availability of data and materials

Data management and monitoring during the study agrees to the rules on protection of personal data. To protect confidentiality, paper-based material (e.g. cognitive test results) will be stored in a secured archive, while electronical data files that are identifiable will be stored in password secured files behind firewall in accordance to regulations. To promote data quality, the primary outcome measurement ($HAMD_{17/6}$ scores) will be obtained during interviews on paper, manually transferred into the local database through LimeSurvey and cross-checked twice before used in analyses. Biological material will be coded with a unique identification-number and access to de-identification keys is restricted to authorized personnel only and stored in a temporary biobank located in secured areas in the laboratory facility. The biomaterial will later be analyzed in batches to reduce noise, and potential extra material after the end of the clinical trial will be transferred to the CIMBI biobank [71]. All biological material will ultimately be anonymized after 15 years after the end of trial.

7. Progress to date

The study opened for inclusion of patients in august 2016. To date, the remaining biological data including genetic status of healthy controls are planned to be collected. Obtained biological material is currently being analyzed and processing of imaging data is on-going. Results from the trial are planned to be communicated to the participants and public through publication in international medical journals.

Discussion

The main purpose of the present study is to identify individual or combined predictors (biomarkers) of standard pharmacological antidepressant treatment outcome in MDD, by using multiple modalities such as brain imaging (PET, fMRI), EEG, cognitive tools, clinical- and molecular markers. Special emphasis in the study design has been given to

evaluate the biomarker 5-HT_4R PET as a promising clinically relevant tool since it 5HT_4R availability is of interest in the pathophysiology and as a target in MDD, and also, it indexes serotonin tonus. The aim of this trial is not to investigate the specific treatment efficacy but to investigate biomarkers for response to standard treatment in a naturalistic setting, e.g., similar to the STAR*D trial [101]. The study includes multiple cross-sectional and longitudinal measures in a large number of patients and controls, which offers a unique opportunity to (a) uncover potential biomarkers or clusters of biomarkers of treatment prediction, (b) apply the findings for stratification of MDD, (c) advance the understanding of pathophysiological underpinnings of MDD, (d) map neurobiological signatures of antidepressant treatment response and lastly (e) to ideally pave a way for a precision medicine approach for optimized treatment of MDD.

Conflict of Interest

VGF and GMK have served as consultants for SAGE therapeutics, GMK has served as a speaker in a Janssen sponsored symposium. MBJ has given talks sponsored by Lundbeck Pharma and Boehringer Ingelheim. Other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

GMK, MBJ and VGF conceived the concept of the study, with help and support from AJ and KKF. KKF, CTI, VNHD, DSS, PMF, MG, HEP, AG and BO have supervised study design and contributed to drafting of the manuscript. All co-authors contributed to, read and approved the final manuscript.

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List of abbreviations

MDD: Major Depressive Disorder
5-HT: 5-hydroxytryptamine (Serotonin)
SSRI: Selective serotonin reuptake inhibitor
SERT: Serotonin transporter
PET: Positron emission tomography
fMRI: Functional magnetic resonance imaging
EEG: Electroencephalogram
$5\text{-HT}_4\text{R}$: Serotonin 4 receptor
rs-fMRI: Resting state functional magnetic resonance imaging
ERP: Event-related potential
LDAEP: Loudness dependence of auditory evoked potentials
CAR: Cortisol awakening response
HPA-axis: Hypothalamic-pituitary-adrenal axis
HAMD_{17}: Hamilton depression rating scale item 17
HAMD_{6}: Hamilton depression rating scale item 6

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Tables

Questionnaires	Time point					
	Baseline	**Week1**	**Week 2**	**Week 4**	**Week 8**	**Week 12**
MINI	X					
HAMD-17/ 6	X	X	X	X	X	X
UKU		X	X	X	X	X
NEO-PIR	X					
CATS	X					
EHI	X					
OS-FHAM	X					
PBI-mother/father	X					
POMS*	X				X	X
Likert-scale*	X				X	X
BDI-II	X			X	X	X
MDI	X			X	X	X
PSS	X			X	X	X
SHAPS	X			X	X	X
RRS	X			X	X	X
CSFQ_F_C	X			X	X	X
SUSY item 32	X			X	X	X
Activity	X			X	X	X
GAD-10	X			X	X	X

Table 1. Table over questionnaires obtained throughout the study. Trait questionnaires at baseline includes personality traits with NEO-PIR [102]; Child Abuse and Trauma Scale (CATS) [103] a survey about early life stress which has shown to be able to modulate the serotonin system in the brain [104]; handedness with Edinburgh Handedness Inventory (EHI) [105]; an in-house version of the Family History Assessment module (FHAM) questionnaire i.e. "Online Stimulant" (OS)-FHAM; Parental Bonding Inventory (PBI) (both mother and father) [106]. State conditions included a self-rating questionnaire of Profile of Mood States (POMS) [107]; an in-house Likert-scale; Beck's Depression Inventory-II (BDI-II) [108]; Major Depression inventory (MDI) [109]; Cohen's Perceived Stress Scale (PSS) [110,111]; Snaith-Hamilton Pleasure Scale (SHAPS) [112]; Rumination Response Scale (RSS) [113]; Changes in Sexual Functioning Questionnaire (CSFQ) [114]; "Sundhed og Sygelighed" Sex Quality Questionnaire item 32 (SUSY-item 32) [115]; an in-house questionnaire about daily physical activity [71] and Generalized Anxiety Disorder-10 (GAD-10) [116]. * Collected in immediate extension to EEG and MR examinations or cognitive testing.

Analysis	Sample	Timepoint		
		Baseline	**Week 8**	**Week 12**
Somatic blood-sample screening	Hemoglobin, white blood cell count, metamyelo.+myelo.+promyelocytes. C-reactive protein.	X	X	X
	Na+, K+, Creatinine	X		
	ASAT, ALAT, GGT, LDH, BAP	X		
	Albumin, Coagulation factors II+VI+X, thrombocytes	X		
	B12, Folate	X		
	25-OH-vitamin D	X		
	Blood sugar, HbA1c	X		
	Triglycerides, total-cholesterol, HDL, LDL	X		
	TSH, Ionized Calcium	X		
	Estradiol, testosterone, progesterone, FSH (females)	X		
Somatic examination	Electro Cardiogram (ECG)	X		
	Neurological status	X		
	Somatic status	X		
Compliance to medicine control	S -escitalopram or S -duloxetine		X	
Biobank	Inflammation and cytokines (hsCRP, TNF-α, IL-6, IL-18 and IL-10)	X	X	X
Biobank	Epigenetics (5-HTT, glucocortocoid-, FKBP5, COMT, MAO-A, estrogen-, oxytocin receptor and oxytocin gene-methylation)	X	X	X
Biobank	Genotypes (rs41271330, 5-HTTLPR, COMT, BDNFval66met)	X		
Biobank	Gene transcription profiles (mRNA and microRNA, ABCB1, FZD7 and WNT2B)	X	X	X
Oxidative stress	Urine (8-oxo-dG and 8-oxo-Guo)	X	X	
Biobank	Saliva (Cortisol awakening response)	X	X	

Table 2. Somatic status and biomaterial assessed at various timepoints throughout the study.

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Paper II

Evidence for a serotonergic subtype of major depressive disorder

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Abstract

Background

First line pharmacological treatment of Major Depressive Disorder (MDD) is selective serotonin reuptake inhibitors (SSRIs), but only 1/3 of patients obtain remission. Cerebral serotonin 4 receptor (5-HT_4R) binding measured with positron emission tomography (PET) is inversely related to serotonin levels and can serve as a proxy for brain serotonin levels. We here determine if a successful outcome of serotonergic treatment in MDD is linked to a serotonergic deficit.

Methods

We [^{11}C]-SB207145 PET-scanned 100 untreated patients with moderate to severe MDD and 91 healthy controls; 40 patients were re-scanned after 8 weeks treatment. All patients were started on SSRI and were followed up clinically after 1, 2, 4, 8 and 12 weeks. Treatment response was measured as change from baseline in Hamilton depression rating scale 6 score.

Results

Before treatment, patients with MDD had 8% lower global 5-HT_4R binding than controls ($p<0.001$). Non-responders did not differ from controls ($p=0.30$), whereas remitters had 9% lower binding than controls at baseline ($p=0.004$). Baseline 5-HT_4R binding did not predict treatment outcome. Independent of treatment outcomes, patients reduced their neostriatum 5-HT_4R binding (-9%, $p<0.001$, N=40) after serotonergic intervention.

Conclusions

Patients with MDD who remit to SSRIs have lower cerebral 5-HT_4R levels than controls whereas non-responders do not differ, suggesting the presence of a serotonergic subtype of MDD. While SSRI intervention decreased neostriatal 5-HT_4R, the change was not associated with the individual clinical outcome. Our data suggest that non-responders to SSRI's constitute a subgroup with non-serotonergic depression.

Trial registration-number: NCT02869035

Introduction

Major depressive disorder (MDD) is one of the most frequent mental disorders worldwide[1] and the lack of predictability of antidepressant drug response remains a tremendous clinical challenge. In clinical practice, trial-and-error drug management directs treatment strategies in lack of biological markers to support pharmacological selection. Currently, the primary pharmacological treatment of MDD is selective serotonin reuptake inhibitors (SSRI) but unfortunately, one third of patients do not achieve remission after an adequate treatment trial.[2] The clinical presentation of MDD is heterogeneous and it is likely that patients diagnosed with MDD fall in subgroups with different etiologies and hence different treatment needs.[3,4] Accordingly, serotonin dysfunction may be etiologically involved in a subset of patients with MDD and only those patients are likely to respond to serotonergic interventions.

Positron emission tomography (PET) allows for studying cerebral serotonin receptor distribution *in vivo* but so far, PET-studies have included limited sample sizes and have not consistently generated firm evidence for serotonergic receptor alterations in MDD,[5,6] or generally been able to predict SSRI treatment response.[7–9] Some,[10,11] but not all,[7] have suggested that cerebral serotonin 1A receptor (5-HT_{1A}R) binding is higher in MDD and one group found that high raphe nuclei 5-HT_{1A}R binding predicts MDD-status in males.[11] Studies of SSRI treatment found that high raphe nuclei 5-HT_{1A}R binding was associated with remission[10] while others found higher 5-HT_{1A}R orbital cortex binding in non-responders.[9]

Preclinical and human studies suggest an involvement of the serotonin 4 receptor (5-HT_4R) in MDD;[12] the 5-HT_4R is a G_s-protein-coupled postsynaptic heteroreceptor which is widely distributed in the brain.[13] In rodents, cerebral 5-HT_4R binding is inversely related to changes in brain serotonin levels,[14–16] and data from healthy individuals PET-scanned with the 5-HT_4R radiotracer [^{11}C]-SB207145 before and after 3 weeks of SSRI or placebo supports that there is an inverse relation to cerebral serotonin levels.[17]

Here, we applied a naturalistic study design and enrolled 100 pharmacologically untreated patients with moderate to severe MDD and investigated them at baseline with [^{11}C]-SB207145 PET and MR neuroimaging before they were started on standard SSRI treatment. Since the clinical effect of SSRIs can be delayed for weeks,[18] we regularly assessed the patients clinically for up to 12 weeks. After 8 weeks of treatment, 43 of the patients were

rescanned with PET and magnetic resonance imaging (MRI). The aims of our study were to investigate if

a) patients with MDD differ in cerebral 5-HT_4R binding at baseline compared to healthy controls

b) cerebral 5-HT_4R binding in patients with MDD predicts remission within 8 weeks after starting SSRI treatment

c) remitted patients with MDD show larger reduction in their cerebral 5-HT_4R binding than non-responders.

Methods

One-hundred antidepressant-free outpatients with moderate to severe MDD were recruited from the mental health system in the capital region of Denmark and included in a non-randomized, 12-week longitudinal, open clinical trial where they received standard antidepressant drug treatment. All participants provided written and informed consent prior to inclusion. Ninety-one healthy, age-and sex-matched volunteers served as baseline controls. The study protocol was approved by all relevant authorities (the Health Research Ethics Committee of the Capital Region of Denmark (H-15017713), the Danish Data Protection Agency (04711/RH-2016-163) and Danish Medicines Agency (EudraCT- 2016-001626-34)). Patients between 18–65 years of age and a Hamilton Depression Rating Scale 17 items ($HAMD_{17}$)[19] score >17 were included. Patients were screened with the Mini International Neuropsychiatric Interview[20] and the diagnosis was confirmed by a specialist in psychiatry. Exclusion criteria were: use of antidepressant medicine within the last two months; duration of the present depressive episode exceeding two years; more than one attempt with an antidepressant treatment in the current episode; previous non-response or known contraindications to an SSRI drug, other primary axis I psychiatric disorder; alcohol/substance abuse or dependence; severe somatic illness; insufficient language skills in Danish; acute suicidal ideation or psychosis; current or planned pregnancy or breast feeding; use of medical treatment affecting CNS (e.g., metoclopramide, ondansetron, serotonergic drugs for migraine, clonidine); contraindications to PET/MRI scans; history of severe brain injury or significant cognitive impediments. Healthy controls were recruited either from our quality-controlled

repository[21] or from an online recruitment site. Ninety-one controls with the same in- and exclusion criteria as the patients (except no past or present psychiatric disorders) were included to match the patients' age and sex as closely as possible. The method and study design are described in detail in Köhler-Forsberg et al (*ref*).

Study assessments for participants and treatment course for patients

Before inclusion, medical history and prior medical treatment was assessed. All participants underwent somatic and psychiatric screening, urine screening for pregnancy or toxicology, and routine blood tests. At baseline, participants were brain scanned with MRI and [^{11}C]-SB207145 PET and 40 of the patients were PET and MRI rescanned at week 8. After completion of the baseline program, patients started antidepressant treatment with escitalopram, individually adjusted to 10-20 mg daily depending on response and side effects. Clinical treatment response was monitored after 1, 2, 4, 8 and 12 weeks of treatment by face-to face visits and $HAMD_{17}$ and $HAMD_6$ ratings.[22] Regular co-ratings between study investigators were implemented. Patients with intolerable side effects or < 25 % reduction from baseline in $HAMD_6$ at week 4 were offered to switch to the serotonin-norepinephrine reuptake inhibitor, duloxetine, individually adjusted (30-120 mg daily). Serum concentration of escitalopram or duloxetine was determined at week 8.

Clinical outcome measures

The primary clinical outcome measure was change in $HAMD_6$ from baseline to week 8. Remitters were defined as having a ≥ 50 % reduction in $HAMD_6$ at week 4 (early responders) and $HAMD_6$ score < 5 at week 8. Non-responders had < 25% reduction in $HAMD_6$ at week 4 (early non-responder) and < 50% reduction in $HAMD_6$ at week 8. Patients in between these categories were referred to as intermediate responders. As a secondary clinical outcome measure, we used relative percentage change in $HAMD_6$ ($r\Delta HAMD_6$) from baseline to week 2, 4, 8 and 12.

PET and MRI procedure

PET/MRI acquisition, pre-processing and PET quantification was performed as previously described (Köhler-Forsberg et al *ref*). Briefly, PET images were acquired during a 120 minutes dynamic scan using a high-resolution research tomography Siemens PET scanner (CTI/Siemens, Knoxville, TN, USA) after intravenous injection of [^{11}C]-SB207145. All patients and 53 controls were scanned with a Siemens 3-Tesla Prisma and 38 controls with a Siemens Magnetom Trio 3-Tesla MR scanner. 3D T1-weighted MRI was co-registered to PET images to obtain structural information. PET scans were motion corrected using the Air 5.2.5 method.[23] PVE-lab was used to extract region of interest (ROIs),[24] delineated on the individuals' MRI. The mean tissue time activity for hemisphere-averaged grey matter volumes was used for kinetic modeling with cerebellum (excluding vermis) as a reference region.[25] The calculated non-displaceable binding potential (BP_{ND}) served as an outcome measure for the 5-HT_4R binding.

Statistics

We included 100 patients to reach a statistical power of 0.8 for detection of a 7% difference in BP_{ND} between remitters and non-responders, with an expected drop-out rate of 20%. For the primary analysis, we used a latent variable model (LVM) to test for global and regional differences in (i) baseline 5-HT_4R BP_{ND} between patients and controls, (ii) baseline 5-HT_4R BP_{ND} between remitters, non-responder, and controls, and (iii) change in BP_{ND} between baseline and follow up (ΔBP_{ND}) and whether ΔBP_{ND} differed between remitters and non-responders. We included neocortex, hippocampus, caudate nucleus and putamen[24] as regions of interest in the LVM because of their relevance in mood disorders.

The receiver operating characteristic (ROC) curve was used to assess if baseline 5-HT_4R BP_{ND} predicted treatment response group. A separate ROC curve was constructed for each brain region and the area under the ROC curve (AUC) was used to summarize the performance of the 5-HT_4R BP_{ND}, 0.5 indicating no discriminative performance.
Secondary analyses included testing with LVM (ii') for an association between baseline BP_{ND} and r$\Delta HAMD_6$, (ii'') for a difference in baseline BP_{ND} between early responder, early non-responder, and healthy controls, (iii') for an association between ΔBP_{ND} and r$\Delta HAMD_6$. In order to assess the data in a more standard manner, analyses (i), (ii), (ii'), and (ii'') were also

performed using multiple linear regressions one for each brain region (neocortex, a limbic region and neostriatum).

All analyses were adjusted for age, sex, injected SB207145 (mass/kg), the 5-HT transporter polymorphism (5-HTTLPR) genotype (L_AL_A or non-L_AL_A), and MR-scanner,[26–29] except within subject rescan-analyses (iii) and (iii') that were only adjusted for the difference in injected SB207145 (mass/kg) between baseline and week 8. When using LVMs, the covariates were included in the measurement model. 5-HT_4R BP_{ND} values were log-transformed. When using LVMs, score tests were used to detect model misspecifications and additional parameters were included until no misspecification could be detected. Missing data in analysis (ii) were handled using complete case analysis. We also adapted an alternative approach where missing values in the primary clinical outcome were imputed based on the clinical outcome at week 4. Nine patients left the study prematurely, those leaving due to early remission were classified as remitters; those leaving because of side effects or suicidality as non-responders. Inverse probability weighting was used to handle other types of dropout using baseline covariates as predictors of dropout. Secondary analyses were performed using complete case analysis. Reported p-values and 95% confidence intervals were two-sided. When performing tests across several brain regions we adjusted p-values (p.adj) and confidence intervals using a single-step Dunnett procedure.[30]

Results

Characteristics of the study participants

Demographics, clinical profile and tracer data (Table 1) showed that patients and controls were comparable, except for injected mass/kg and a minor difference in education. We included 91 patients for baseline analyses and 78 in the longitudinal analyses; of the latter, 22 were remitters and 13 non-responders after 8 weeks, and 34 were early responders and 14 early non-responders after 4 weeks. Table S-1 describes baseline psychopathological profile for non-responders and remitters. Six patients switched to duloxetine before week 8. Re-scan data was obtained from 12 remitters, 5 non-responders and 23 responders. No serious adverse events occurred during the study. Remission rate was 48% at week 12 according to remission-criteria used in, e.g., STAR*D study ($HAMD_{17} \leq 7$)[31] and comparable to similar clinical trials.[32] Figure S-1 shows the CONSORT diagram.

Baseline differences in 5-HT_4R BP_{ND} between patients and controls.

We found 7-8% lower regional BP_{ND} in untreated patients with MDD compared to controls ($p < 0.001$), (Figure 1 and S-2). Linear regression models generated the same outcome (Table S-2). Since BP_{ND} in the caudate nucleus and putamen were especially correlated, these regions were pooled into "neostriatum" for the subsequent analyses.

Treatment outcome and prediction analysis

Global BP_{ND} was lower in remitters than in controls ($p=0.004$, Table 2), with 8-10% lower binding in neocortex (Figure 2), hippocampus and neostriatum. Figure S-3 displays the baseline BP_{ND} for patients according to clinical response group and controls. There was no difference in global BP_{ND} between non-responders and controls ($p=0.31$) or between remitters and non-responders ($p=0.18$). Handling missing data using a combination of imputation and inverse probability weighting lead to estimates and conclusions that were similar to the complete case analysis (Table S-3).

Response categories at week 4 (Table 2) showed 8-10% lower BP_{ND} in early responders than in controls ($p=0.002$), and 7-9% lower BP_{ND} in early responders compared to early non-responders ($p=0.046$). There was no difference between early non-responders and controls ($p=0.79$). Similar results were found when using multiple linear regression (Table S-4). Further, we found a correlation between baseline BP_{ND} and $r\Delta HAMD_6$ at week 4 ($p=0.03$), but not at week 8 ($p=0.98$). Univariate analysis identified the correlation in neocortex at week 4 only (Table S-5).

Regional baseline BP_{ND} did not show discriminative power for identifying non-responders from remitters: neocortex (AUC: 0.63, $p=0.20$), limbic region (AUC 0.57, $p=0.54$), neostriatum (AUC: 0.57, $p=0.52$).

Rescan analysis

Eight weeks after initiating SSRI treatment, patients showed a decrease in global BP_{ND} compared to baseline ($p<0.001$, LVM-model, N=40). At a regional level, the decrease in BP_{ND} constituted 9.0% [-12.8%; -5.0%] in neostriatum ($p.adj<0.0001$) but no significant change was seen in neocortex (-1.4% [-6.2%; 3.6%], $p.adj=0.79$) or hippocampus (-1.7% [-

7.5%; 4.5%], p.adj=0.80) (Figure 3 and S-4). The decline was not associated with categorical response at week 8 (p=0.60) or r$\Delta HAMD_6$ (p=0.74).

Discussion

In this to date largest single clinical PET trial investigating the serotonin system in MDD, we show that antidepressant-free patients with a moderate to severe major depressive episode on average had 7-8% lower cerebral 5-HT_4R binding than healthy controls. Intriguingly, patients who remitted after 4 or 8 weeks of serotonergic medication had 8-10% lower cerebral 5-HT_4R baseline binding whereas non-responders did not differ from controls. When patients were PET-rescanned 8 weeks after starting SSRI treatment, their striatal 5-HT_4R binding had decreased, irrespective of the clinical treatment outcome. These results support the notion that only a subgroup of patients with MDD have a serotonergic dysfunction and that accordingly patients within this subgroup are effectively treated with SSRI.

Our finding of abnormally low 5-HT_4R binding in the subgroup of unmedicated MDD patients that remit on SSRI treatment could constitute a trait or a state feature. A previous study reported a negative association between the number of first degree relatives with MDD and striatal 5-HT_4R binding in healthy individuals,[33] and it was suggested that low 5-HT_4R binding could be a trait marker for increased risk of MDD, possibly reflecting increased cerebral serotonin levels that ensured euthymia. Since we now find a lower global 5-HT_4R binding specifically in patients that remit in response to SSRI treatment, it seems less likely that low 5-HT_4R binding is a *general* trait marker for unmedicated MDD. With the observed inverse relation between 5-HT_4R binding and cerebral serotonin levels, [14–17] one interpretation is that already prior to treatment, remitters have higher brain serotonin levels. Increased serotonin levels could be the brain's attempt to maintain euthymia, and addition of serotonergic acting drugs increases serotonin levels sufficiently for remission to occur. Alternatively, or in combination, patients responding to SSRIs may be genetically predisposed for low cerebral 5-HT_4R density.

Our observation that striatal 5-HT_4R binding decreases in response to increased serotonin levels also in patients with MDD (Figure S-5) is consistent with observations in preclinical studies and in healthy indivdiuals[14–17]. After 8 weeks of treatment, we found across response-groups a 9% decrease in neostriatum 5-HT_4R binding, suggesting that it was not a failure of

the drug effect on brain serotonin levels that explained a poor clinical drug response. Interestingly, whereas the reduction in 5-HT_4R binding seen after serotonergic treatment was specific for neostriatum, differences between patients with MDD and controls showed a global effect across all brain regions. The regional difference in the rescan data could be due to the drug intervention having a specific effect by increasing serotonin in neostriatum[16] which together with thalamus is massively innervated by serotonergic fibers and has among the highest density of serotonin transporters.[13]

Short-term administration of 5-HT_4R agonists to rodents generates rapid antidepressant/anxiolytic-like behavior,[34,35] hippocampal neurogenesis,[36] prophylactic antidepressant and anxiolytic characteristics[37] and the first translational study recently confirmed enhanced memory effects in healthy volunteers.[38] It remains to be tested in clinical trials if 5-HT_4R agonists could constitute a new therapeutic target for the MDD serotonergic subtype patients. Whereas cerebral 5-HT_4R binding cannot be used as the sole biomarker for prediction of SSRI treatment outcome, our data opens for an interesting possibility of identification of a distinct biological subtype within MDD with a "non-serotonergic"-related depression. Such a subgroup would be amenable for investigation of non-serotonergic drug effects.

In conclusion, we here provide novel support that MDD patients with a primary serotonergic dysfunction constitute a subgroup where SSRI treatment is particularly effective. Neuroimaging of the 5-HT_4R can thus be regarded as a biomarker that aids to identify subgroups of patients with MDD (e.g., non-serotonergic related depression) which may enable future precision medicine approaches.

Author contributions:

VGF, MBJ, GMK, and AJ wrote the study protocol. KKF, SVL, ASP, EL, VHD, CI, AJ, MM, HE, VGF, and MBJ participated in clinical assessments. CS and KKF performed imaging analyses. BO and KKF performed the statistical analyses. KKF, GMK and VGF drafted the manuscript. All authors critically reviewed the manuscript and approved the final content.

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Disclosure

GMK has received honoraria as expert advisor for Sage Therapeutics and as speaker for Jannsen. VGF has served as consultant for SAGE therapeutics. MBJ has given talks sponsored by Boehringer Ingelheim and Lundbeck Pharma. All other authors declare no conflict of interest.

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Tables

		Patients with MDD			Healthy controls		
		n	**%**		**n**	**%**	**p-value** [a]
Sex	Female	65	71.4		55	60.4	0.16
	Male	26	28.6		36	39.6	
5-HTTLPR genotype	L_AL_A	26	28.6		27	29.7	1
	Non-L_AL_A	65	71.4		64	70.3	
	Range	**n**	**Mean (SD)**	**Range**	**n**	**Mean (SD)**	**p-value** [b]
Age (years)	18.3-57.3	91	27.1 (8.2)	19.2-60.1	91	27.1 ± 8.0	0.57
Years of education	5-12	76	11.6 (1.1)	9-12	91	11.9 (0.5)	**0.003**
BMI (kg/m^2)	17.1-45.1	91	24.5 (5.6)	18.3-36.9	91	23.6 (3.1)	0.96
$HAMD_{17}$	18-31	91	22.9 (3.4)	NA		NA	NA
$HAMD_6$	7-17	91	12.3 (1.6)	NA		NA	NA
MDI	16-50	89	34.7 (7.2)	0-18	91	5.6 (4.2)	**< 0.001**
Injected dose (MBq)	263.0-615.0	91	577.4 (56.0)	226-617	91	569.4 (76.3)	0.20
Injected mass/kg (µg/kg)	0.004-0.082	91	0.013 (0.015)	0.003-0.07	91	0.017 (0.015)	**0.028**
Cerebellum, area under curve (kBq/ml)	3.9-17.8	91	10.3 (2.6)	3.2-16.2	85	10.3 (2.5)	0.75

Table 1. Clinical profile, demographic and radiotracer data for patients with MDD and controls at baseline. BMI: body mass index. $HAMD_{17/6}$: Hamilton depression rating scale 17 or 6 items. MDI: Major depressive inventory. NA: not applicable. [a] p-value computed using a Fisher's exact t-test, [b] p-value computed using a Mann Whitney U-test.

	Early responder vs. controls	Early non-responder vs. controls	Early responder vs. early non-responder	Remitter vs. control	Non-responder vs. control	Remitter vs. non-responder
n	34 vs. 91	14 vs. 91	34 vs. 14	22 vs. 91	13 vs. 91	22 vs. 13
Week	4	4	4	8	8	8
Global effect						
p	**0.002**	0.79	**0.046**	**0.004**	0.31	0.18
Regional effect						
Neocortex	-8.96% [-14.63; -2.91]	-1.03% [-8.37;6.9]	-8.01% [-15.61;0.27]	-9.5% [-15.85; -2.66]	-3.89% [-11.16; 3.98]	-5.84% [-14.04; 3.16
Hippocampus	-10% [-16.27; -3.25]	-1.16% [-9.34; 7.77]	-8.94% [-17.34; 0.31]	-9.92% [-16.54; -2.77]	-4.07% [-11.65;4.17]	-6.1% [-14.65; 3.31]
Neostriatum	-7.68% [-12.6; -2.47]	-0.88% [-7.17; 5.84]	-6.86% [-13.45; 0.23]	-8.19% [-13.76; -2.27]	-3.34% [-9.64;3.4]	-5.02% [-12.16;2.7]

Table 2. Cerebral 5-HT$_4$R binding in controls and in MDD, according to treatment response at week 4 and 8. The p-values refers to the testing of BP_{ND} between two groups across all regions. The last three rows display the region-specific difference in BP_{ND} between two groups with confidence interval, corrected for 3 comparisons (i.e. across the 3 regions). All estimates originate from the LVM.

Figures

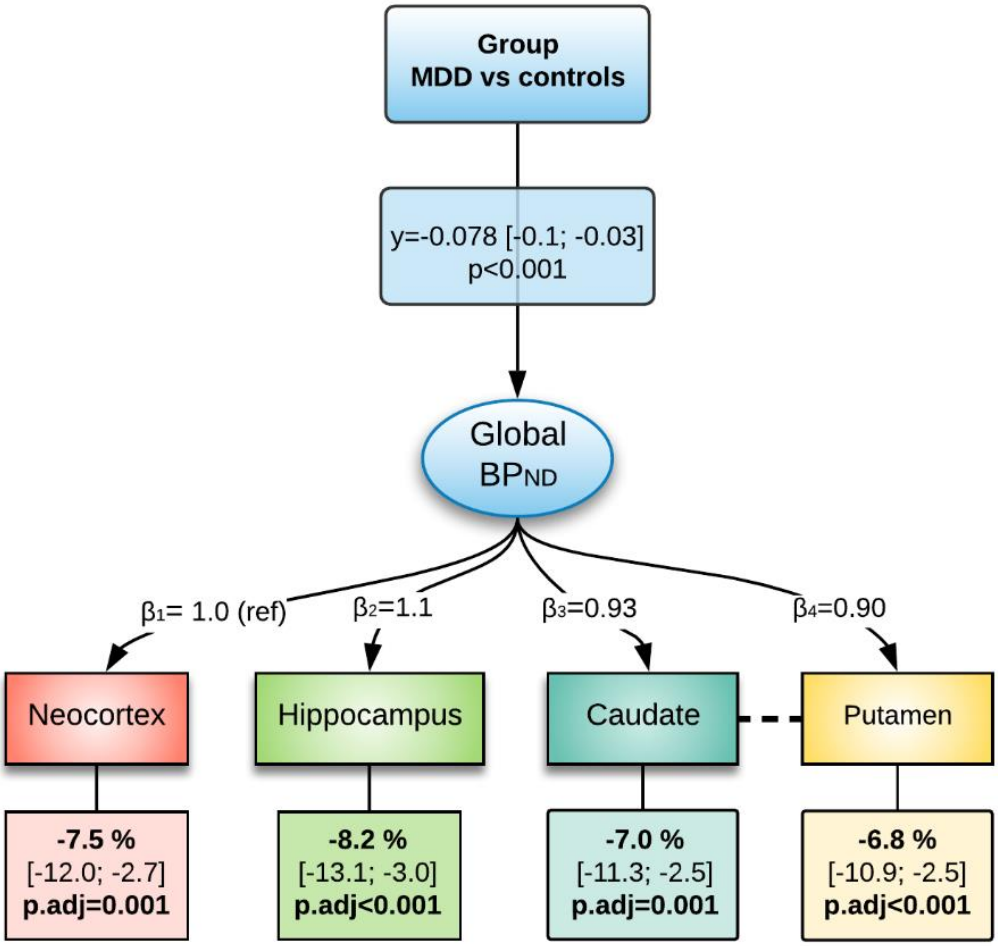


Figure 1. Estimated latent variable model for the 5-HT4R binding in untreated patients with MDD and controls. γ is the effect of group-status on the global (log-transformed) BP_{ND}, β is the loading, the dashed line indicates additional shared correlations between caudate nucleus and putamen. The lower boxes indicate, for each brain region, the percentage difference in baseline 5-HT_4R binding between MDD and controls (p-values and confidence intervals are adjusted for 4 comparisons). Age, sex, 5-HTTLPR gene-status, MR-scanner type and injected mass/kg are included as covariates in the model.

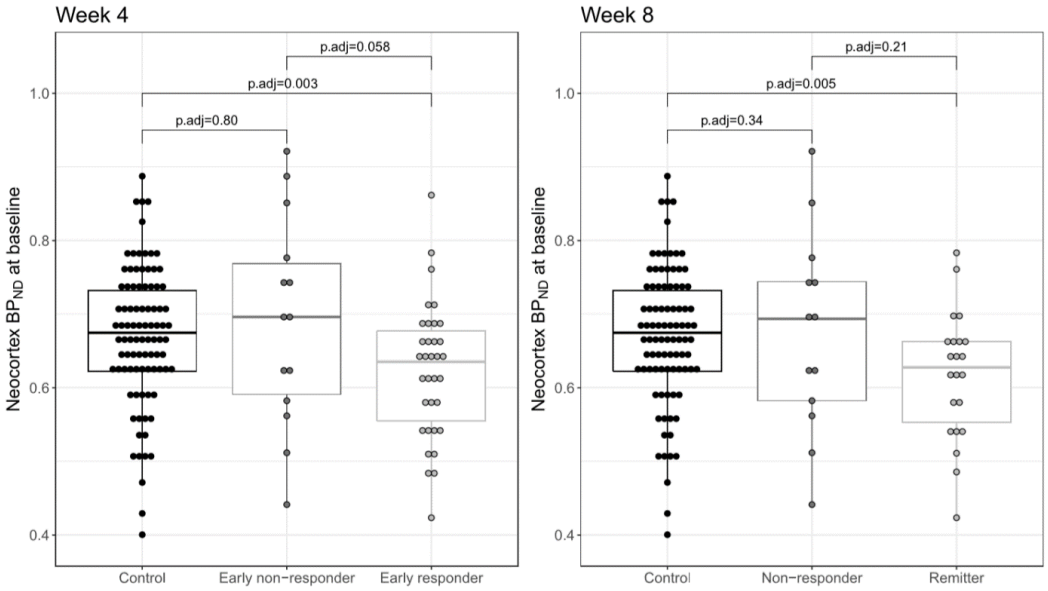
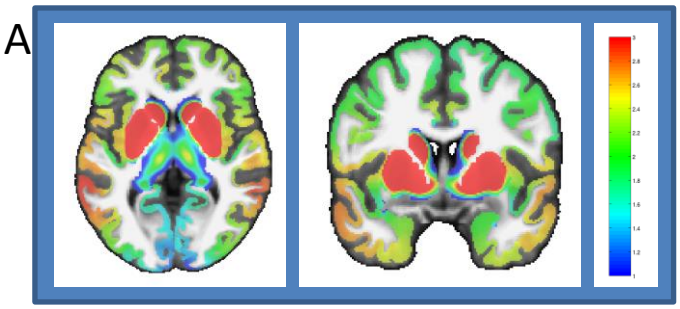


Figure 2. **Scatter plot of 5-HT$_4$R baseline binding in neocortex for healthy controls and patients with MDD according to clinical outcome at week 4 and 8.** Week 4: Controls (n=91), early responders (n=34), and early non-responders (n=14). Week 8: Controls (n=91), remitters (n=22), and non-responders (n=13). P-values originate from the latent variable model and were adjusted for 3 comparisons.



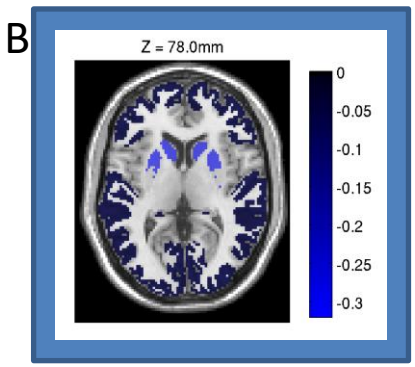


Figure 3. Panel A. Average density maps (pmol/ml) for the 5-HT$_4$R at baseline in patients with MDD (n=91). Atlas used from Beliveau and colleagues.[13] **Panel B.** Difference in mean 5-HT$_4$R binding from baseline and rescan in patients (N=40). Regions of interest for the latent variable model (neocortex, hippocampus and neostriatum) are shown. The post-SSRI effect was most prominent in neostriatum (lighter blue).

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Supplementary material

Supplementary Tables

	Remitter (n=22)		Non-responder (n=13)	
	Range	*Mean (SD)*	*Range*	*Mean (SD)*
$HAMD_{17}$ baseline	18-29	22.9 (3.0)	18-27	21.5 (2.1)
$HAMD_6$ baseline	8-14	11.9 (1.5)	7-14	11.2 (1.8)
$HAMD_6$ items				
- **Depressed mood**	2-4	2.7 (0.6)	2-4	3.1 (0.5)
- **Feelings of guilt**	0-3	1.6 (0.7)	0-3	1.5 (0.8)
- **Work and activities**	1-4	2.3 (0.7)	2-3	2.3 (0.5)
- **Retardation**	0-3	1.2 (0.8)	0-2	0.5 (0.8)
- **Anxiety (psychic)**	1-4	2.5 (0.7)	0-3	2 (0.8)
- **Somatic symptoms (general)**	0-2	1.6 (0.7)	0-2	1.8 (0.6)
MDI baseline	16-50	32.9 (9.0)	29-45	35.9 (5.9)*

Table S-1. Descriptive table of baseline psychopathology in non-responders and remitters. $HAMD_6$ items are specified. *One patient failed to fill out MDI at baseline. MDI: Major Depression Inventory. $HAMD_{17/6}$: Hamilton Depression Rating Scale 17 and 6 items. Group differences tested using a Mann Whitney U-test.

ROI	% change in BP_{ND} (MDD vs control)	Standard error	95% CI	p.adj
Neocortex	-8.86	2.31	-14.00; -3.41	<0.001
Limbic region	-6.53	2.14	-11.42; -1.37	0.011
Neostriatum	-6.21	2.16	-11.15: -0.99	0.017

Table S-2. Regional percentage difference in 5-HT_4R binding in MDD versus controls at baseline. Estimated by multiple linear regressions adjusted for age, sex, 5-HTTLPR status, injected tracer (mass/kg) and MR scanner type. P-values are adjusted for multiple comparison.

	Remitter vs. Control (ref)	Non-responder vs. Control (ref)	Remitter vs. non-responder (ref)
n	23 vs. 91	16 vs. 91	23 vs. 16
week	8	8	8
p	**0.01**	0.34	0.22
Neocortex	-8.11% [-14.33;-1.42]	-3.31% [-9.96;3.83]	-4.96% [-12.62;3.37]
Hippocampus	-8.39% [-14.82;-1.47]	-3.42% [-10.30;3.98]	-5.14% [-13.05;3.50]
Neostriatum	-6.95% [-12.37;-1.20]	-2.83% [-8.55;3.26]	-4.25% [-10.87;2.87]

Table S-3. Cerebral 5-HT$_4$R binding in controls and in MDD according to treatment outcome in week 8 after accounting for missing data. The p-values reflect the difference in BP_{ND} between two groups across all regions. The last three rows display the region-specific difference in binding between two groups with confidence intervals, corrected for 3 comparisons (i.e. across the 3 regions). All estimates originate from the LVM after imputation and inverse probability weighting.

	Early responder vs. control (ref)	Early non-responder vs. control (ref)	Early responder vs. early non-responder (ref)	Remitter vs. control (ref)	Non-responder vs. control (ref)	Remitter vs. non-responder (ref)
n	34 vs 91	14 vs 91	34 vs 14	22 vs 91	13 vs 91	22 vs 13
Week	4	4	4	8	8	8
Neocortex	-7.95% -14.49; -0.91]	2.16% [-8.07;13.53]	-9.9% [-20.21;1.73]	-8.52% [-16.37;0.08]	-0.06% [-10.55; 11.65]	-8.46% [-20.13;4.92]
p.adj	**0.025**	0.85	0.10	0.052	1.00	0.25
Limbic region	-4.48%	2.79%	-7.07%	-4.08%	0.13%	-4.21%
p.adj	[-11.27;2.84]	[-7.63;14.38]	[-17.92;5.21]	[-11.81;4.32]	[-9.74;11.09]	[-15.72;8.87]
	0.28	0.77	0.30	0.44	1.00	0.67
Neostriatum	-4.92% [-11.14;1.73]	0.93% [-8.47;11.3]	-5.8% [-15.91;5.52]	-5.25% [-13.01;3.2]	-1.42% [-11.35;9.61]	-3.89% [-15.76;9.67]
p.adj	0.17	0.97	0.37	0.27	0.94	0.73

Table S-4. Association between baseline 5-HT$_4$R binding and categorical response group week 4 and week 8 using linear regression. Adjusted for age, sex, 5-HTTLPR status, MR-scanner type and injected tracer (mass/kg).

Region	Week	Partial correlation	Confidence Interval	p	p.adj
Neocortex	2	0.19	[-0.04;0.41]	0.10	0.18
	4	0.31	[0.09;0.52]	**0.005**	**0.01**
	8	0.09	[-0.14;0.31]	0.44	0.64
	12	0.05	[-0.19;0.28]	0.70	0.91
Limbic region	2	0.08	[-0.14;0.30]	0.47	0.71
	4	0.22	[0.003;0.44]	**0.047**	0.09
	8	0.01	[-0.21;0.24]	0.90	1.00
	12	-0.02	[-0.24;0.21]	0.88	0.99
Neostriatum	2	0.06	[-0.17;0.28]	0.62	0.86
	4	0.14	[-0.08;0.36]	0.21	0.35
	8	-0.03	[-0.26;0.20]	0.79	0.96
	12	0.03	[-0.20;0.25]	0.83	0.98

Table S-5. Correlation between baseline 5-HT_4R binding and percentage change in $HAMD_6$ at week 2-12, using partial correlation correction. Covariates: age, sex, 5-HTTLPR status and injected tracer (mass/kg).

Supplementary Figures

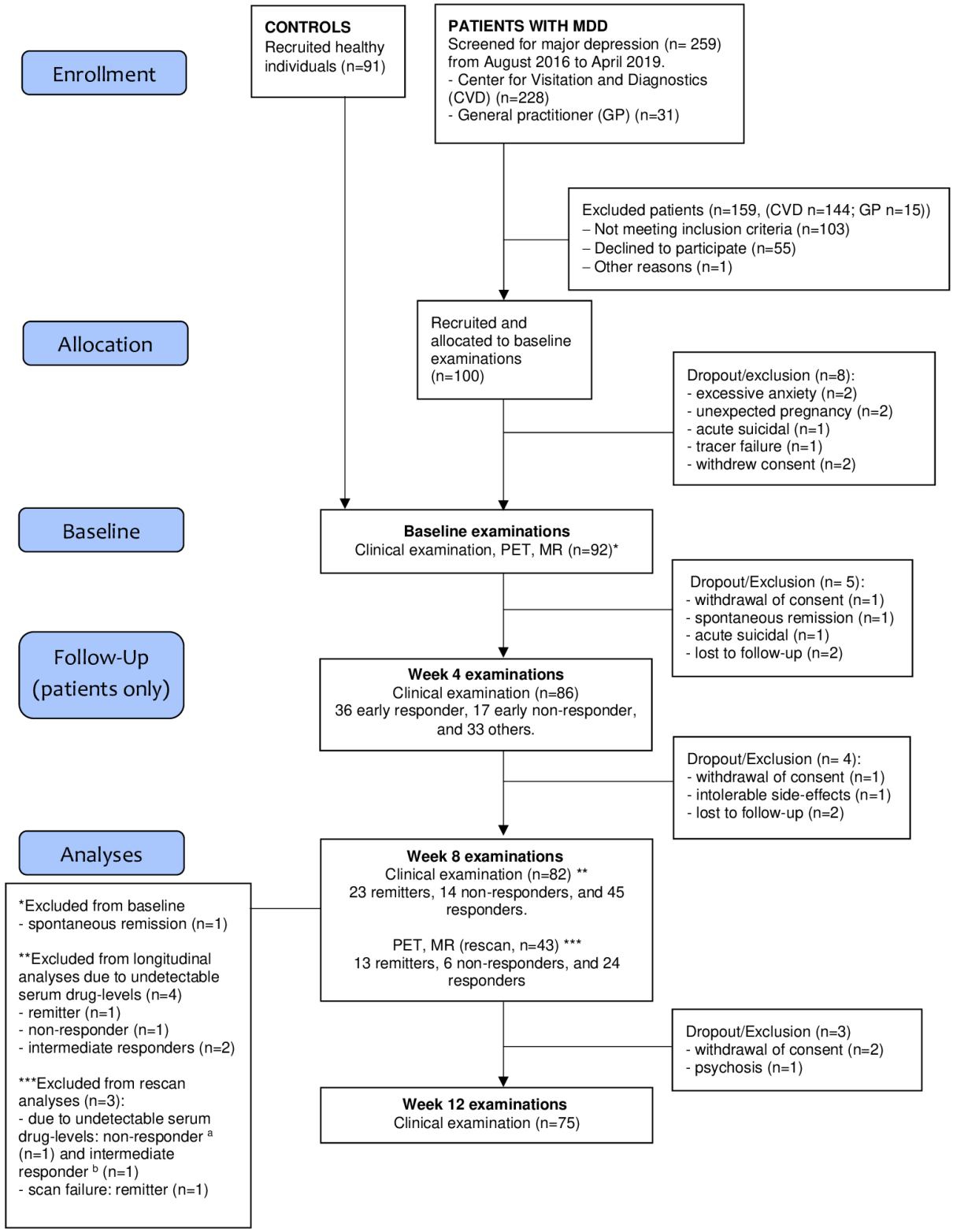

Figure S-1. CONSORT flow diagram over study participants. *One patient was excluded from baseline analyses because of spontaneous remission. **Four patients were excluded from the analyses at week 4, 8, and 12 because of undetectable serum-levels of escitalopram or duloxetine. *** Three

patients were excluded from rescan analyses because of scanner failure and undetectable serum-levels of [a] duloxetine or [b] escitalopram.

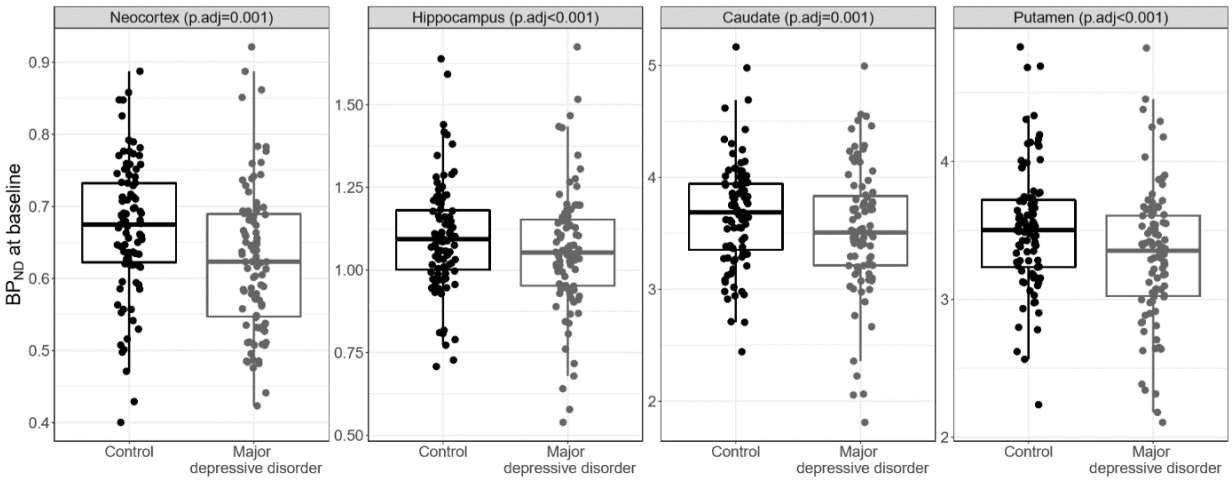

Figure S-2 Scatter plot of regional cerebral baseline 5-HT$_4$R binding in patients with MDD and controls.

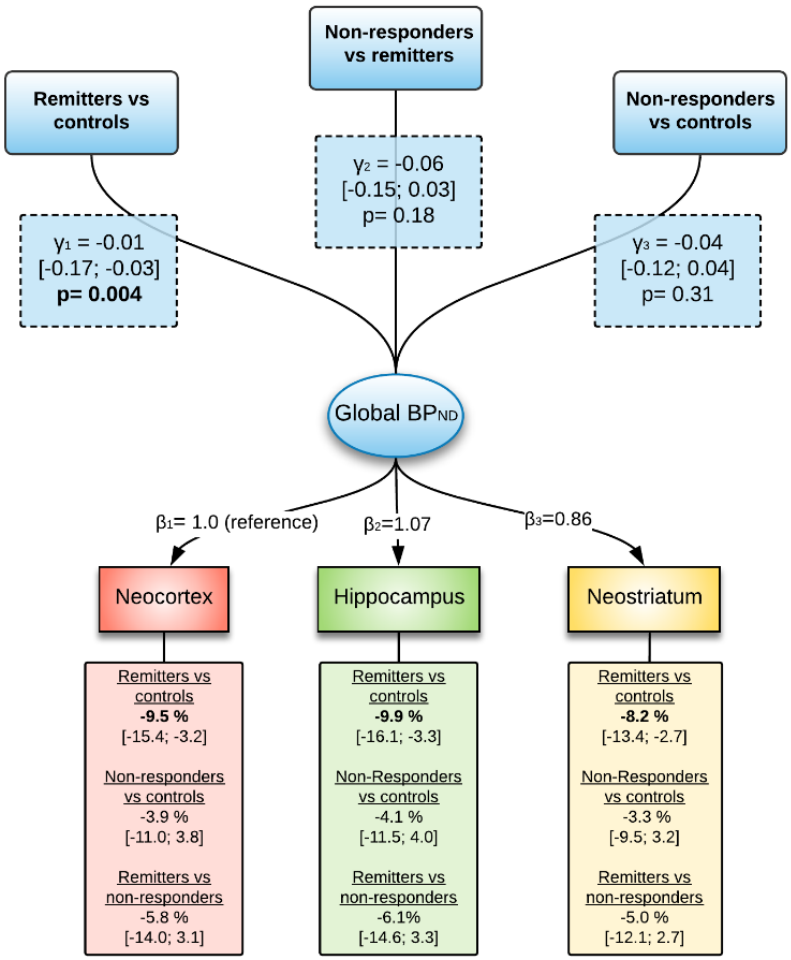

Figure S-3. Latent variable model of differences in 5-HT$_4$R binding at baseline in controls and MDD, according to treatment response at week 8. γ is the effect of response-status on the global (log-transformed) BP_{ND}, β is the regional loading. The lower boxes indicate, for each brain

region, percentage differences in baseline 5-HT$_4$R binding between each response group and controls (p-values and confidence intervals are adjusted for 4 comparisons). The model includes the following covariates: age, sex, 5-HTTLPR gene-status, MR-scanner type and injected mass/kg.

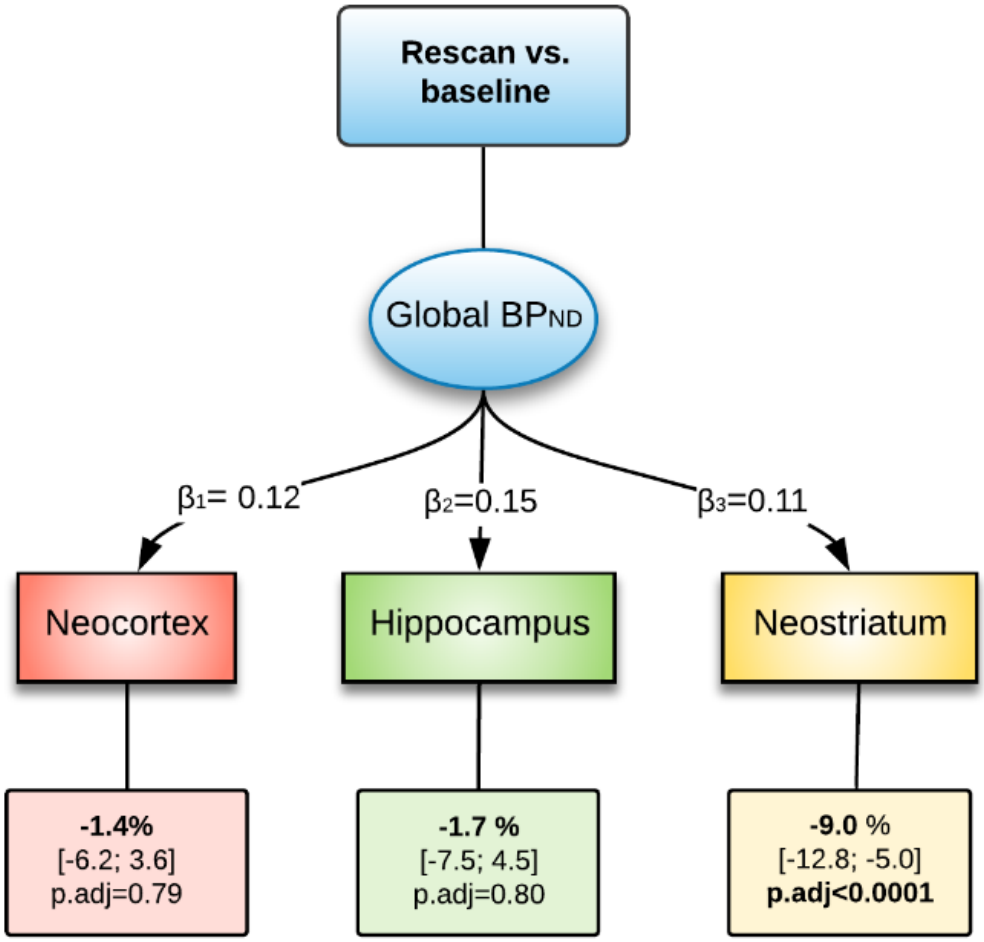

Figure S-4. Latent variable model of changes in 5-HT$_4$R BP$_{ND}$ from baseline to rescan at week 8. β is the regional loading. The lower boxes indicate, for each brain region, the percentage difference in 5-HT$_4$R binding from baseline to rescan. p-values and confidence intervals are adjusted for multiple comparisons. Injected SB207145 (mass/kg) was included in the model as a covariate.

Supplementary text for Figure S-5.

In a post hoc analysis, we were able to replicate the findings from Haahr et al [17]. Using the same analysis strategy, we found a decrease in global binding (-0.07, 95% CI [-0.11 to -0.04], p=<0.001), see Figure S-5. Since we adapted a naturalistic design, we cannot directly know to what extent the decline in neostriatum 5-HT_4R binding is any larger than that seen in healthy controls [17], but the observation is a replication and extension of these prior findings and supports that long-term SSRI intervention increases brain serotonin levels also in patients with MDD.

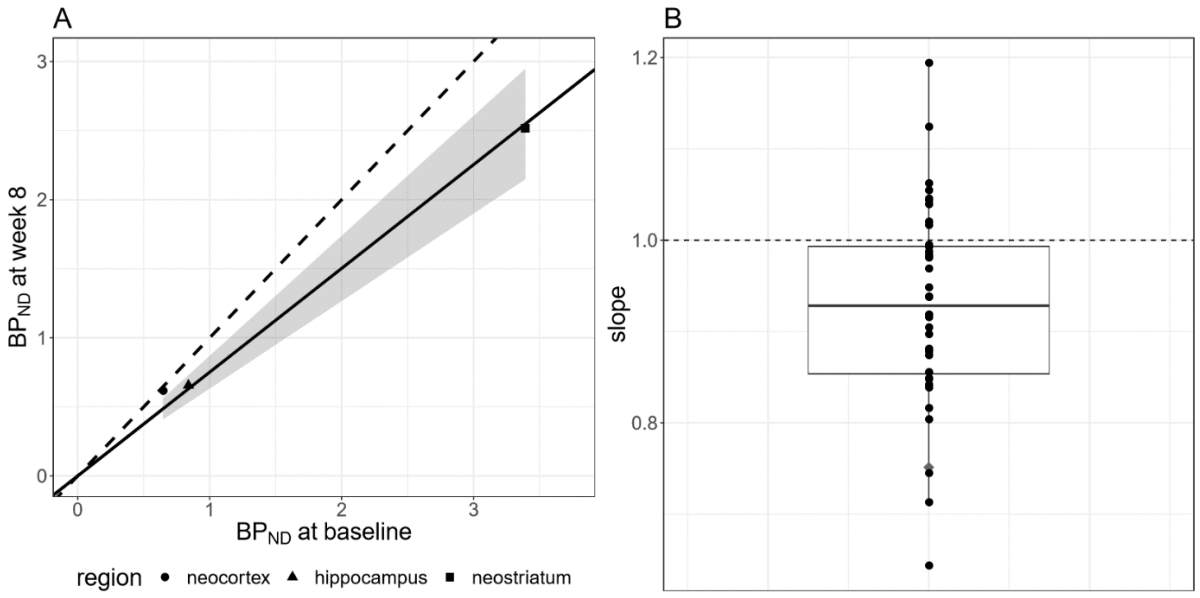

Figure S-5. Left panel: estimated regression line (black line) between the BP_{ND} at baseline and at week 8 across three brain regions for a representative patient. The shaded area indicates the 95% confidence interval for the regression line and the dotted line the identity line (values below the dashed line indicates a decrease in BP_{ND}). Right panel: Boxplot with dots representing the individually estimated slopes of the regression line.

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PHD-THESIS
DECLARATION OF CO-AUTHORSHIP

The declaration is for PhD students and must be completed for each conjointly authored article. Please note that if a manuscript or published paper has ten or less co-authors, all co-authors must sign the declaration of co-authorship. If it has more than ten co-authors, declarations of co-authorship from the corresponding author(s), the senior author and the principal supervisor (if relevant) are a minimum requirement.

1. Declaration by	
Name of PhD student	Kristin Linn Köhler-Forsberg
E-mail	forsberg.kristin@gmail.com
Name of principal supervisor	Martin Balslev Jørgensen
Title of the PhD thesis	The serotonin 4 receptor binding as a novel imaging marker in major depressive disorder and the association to antidepressant treatment response.

2. The declaration applies to the following article	
Title of article	Predicting treatment outcome in Major Depressive Disorder using serotonin 4 receptor PET brain imaging, functional MRI, cognitive-, EEG-based and peripheral biomarkers: a NeuroPharm open label clinical trial protocol
Article status	
Published ☐ Date:	Accepted for publication ☐ Date:
Manuscript submitted ☒ Date: November 28 2019	Manuscript not submitted ☐
If the article is published or accepted for publication, please state the name of journal, year, volume, page and DOI (if you have the information).	

3. The PhD student's contribution to the article *(please use the scale A-F as benchmark)* Benchmark scale of the PhD-student's contribution to the article **A.** Has essentially done all the work (> 90 %) **B.** Has done most of the work (60-90 %) **C.** Has contributed considerably (30-60 %) **D.** Has contributed (10-30 %) **E.** No or little contribution (<10 %) **F.** Not relevant	**A, B, C, D, E, F**
1. Formulation/identification of the scientific problem	E
2. Development of the key methods	F
3. Planning of the experiments and methodology design and development	C
4. Conducting the experimental work/clinical studies/data collection/obtaining access to data	B
5. Conducting the analysis of data	F
6. Interpretation of the results	F
7. Writing of the first draft of the manuscript	A
8. Finalisation of the manuscript and submission	A

Provide a short description of the PhD student´s specific contribution to the article.[i]
The article is a trial study protocol with > 10 co-authors. The original protocol was written before the PhD student was enrolled. The PhD student has foremost contributed with the method-section through practical planning and set-up of the clinical trial including recruitment of patients, development of case report files for the patients, details of the condution of data collection throughout the study, has followed patients over time and performed examinations for a majority of all included participants, has been responsible for registration of the trial as a clinical trial, had contact with authorities and the monitoring unit (GCP). The student has been writing the first draft of the paper and finalized the manuscript together with the senior author. She also prepared and submitted the article to a journal.

4. Material from another thesis / dissertation[II]	
Does the article contain work which has also formed part of another thesis, e.g. master's thesis, PhD thesis or doctoral dissertation (the PhD student's or another person's)?	Yes: ☐ No: ☒
If yes, please state name of the author and title of thesis / dissertation.	
If the article is part of another author's academic degree, please describe the PhD student's and the author's contributions to the article so that the individual contributions are clearly distinguishable from one another.	

5. Signatures of the co-authors[III]				
	Date	Name	Title	Signature
1.	9.4.20	Vibe Gedsø Frøkjær (senior author)	MD, PhD	
2.	30.3.20	Martin Balslev Jørgensen (principal supervisor)	MD, ~~PhD~~, DMSc	MBJ
3.	31.3.20	Gitte Moos Knudsen (corresponding author)	MD, PhD, DMSc	
4.				
5.				
6.				
7.				
8.				
9.				
10.				

6. Signature of the principal supervisor
I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge. Date: 30.3.20 Principal supervisor: Martin Balslev Jørgensen MBJ

7. Signature of the PhD student
I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge. Date: 03-28-2020 PhD student: Kristin Linn Köhler-Forsberg

Please learn more about responsible conduct of research on the Faculty of Health and Medical Sciences' website.

[i] This can be supplemented with an additional letter if needed.
[ii] Please see Ministerial Order on the PhD Programme at the Universities and Certain Higher Artistic Educational Institutions (PhD Order) § 12 (4):
"Any articles included in the thesis may be written in cooperation with others, provided that each of the co-authors submits a written declaration stating the PhD student's or the author's contribution to the work."
[iii] If more signatures are needed please add an extra sheet.

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PHD-THESIS
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The declaration is for PhD students and must be completed for each conjointly authored article. Please note that if a manuscript or published paper has ten or less co-authors, all co-authors must sign the declaration of co-authorship. If it has more than ten co-authors, declarations of co-authorship from the corresponding author(s), the senior author and the principal supervisor (if relevant) are a minimum requirement.

1. Declaration by	
Name of PhD student	Kristin Linn Köhler-Forsberg
E-mail	forsberg.kristin@gmail.com
Name of principal supervisor	Martin Balslev Jørgensen
Title of the PhD thesis	The serotonin 4 receptor binding as a novel imaging marker in major depressive disorder and the association to antidepressant treatment response.

2. The declaration applies to the following article	
Title of article	Evidence for a serotonergic subtype of major depressive disorder.
Article status	
Published ☐ Date:	Accepted for publication ☐ Date:
Manuscript submitted ☐ Date:	Manuscript not submitted ☒
If the article is published or accepted for publication, please state the name of journal, year, volume, page and DOI (if you have the information).	

3. The PhD student's contribution to the article *(please use the scale A-F as benchmark)* Benchmark scale of the PhD-student's contribution to the article **A.** Has essentially done all the work (> 90 %) **B.** Has done most of the work (60-90 %) **C.** Has contributed considerably (30-60 %) **D.** Has contributed (10-30 %) **E.** No or little contribution (<10 %) **F.** Not relevant	**A, B, C, D, E, F**
1. Formulation/identification of the scientific problem	E
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4. Conducting the experimental work/clinical studies/data collection/obtaining access to data	B
5. Conducting the analysis of data	B
6. Interpretation of the results	B
7. Writing of the first draft of the manuscript	A
8. Finalisation of the manuscript and submission	B

Provide a short description of the PhD student´s specific contribution to the article.[i]

The article has > 10 co-authors. The PhD student has foremost contributed with the method-section through practical planning and set-up of the clinical trial including recruitment of patients, development of case report files for the patients, details of the condution of data collection throughout the study, has followed patients over time and performed examinations for a majority of all included participants, has been responsible for registration of the trial as a clinical trial, had contact with authorities and the monitoring unit (GCP). The student has performed the statistical analyses together with the second author, has been writing the first draft of the paper and finalized the manuscript together with the senior authors. She has also prepared and will submit the article to a peer-reviewed scientific journal of high impact.

4. Material from another thesis / dissertation[II]	
Does the article contain work which has also formed part of another thesis, e.g. master's thesis, PhD thesis or doctoral dissertation (the PhD student's or another person's)?	Yes: ☐ No: ☒
If yes, please state name of the author and title of thesis / dissertation.	
If the article is part of another author's academic degree, please describe the PhD student's and the author's contributions to the article so that the individual contributions are clearly distinguishable from one another.	

5. Signatures of the co-authors[III]

	Date	Name	Title	Signature
1.	9.4.20	Vibe Gedsø Frøkjær (senior author)	MD, PhD	
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6. Signature of the principal supervisor

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.
Date: 30.3.20
Principal supervisor: Martin Balslev Jørgensen MBJ

7. Signature of the PhD student

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.
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PhD student: Kristin Linn Köhler-Forsberg

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