Genetic correlates of the cortisol awakening response in patients remitted from major depressive disorder

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List of abbreviations:

^{2}H	Deuterium
5-HT	Serotonin
5-HTT	Serotonin transporter
5-HTTLPR	Serotonin -transporter-linked polymorphic region
A/G	Adenine/Guanine
AA	Amino acid
ACTH	Adrenocorticotropic hormone
AUC	Area under the curve
AVP	Arginine vasopressin
BDNF	Brain-derived neurotrophic factor
bp	Base pair
cAMP	Cyclic-adenosine monophosphate
CAR	Cortisol awakening response
CBG	Corticosteroid binding globulin
CRF	Corticotropin releasing hormone
CRF1	Corticotropin releasing factor receptor 1
CRF2	Corticotropin releasing factor receptor 2
CYP11B1	Cytochrome p450 family 11 subfamily B member 1
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
Fwd	Forward
GC	Glucocorticoids
GPCR	G-protein coupled receptor
GRE	Glucocorticoid responsive element
HPA	Hypothalamus- pituitary- adrenal gland
IL	Intracellular loop
L176M	Leucine176Methionine
NS	Not significant
MC	Mineralocorticoid
MCR	Mineralocorticoid receptor
MC1R	Melanocortin 1 receptor

MC2R	Melanocortin 2 receptor
MDD	Major depressive disorder
MRAP	Melanocortin 2 receptor accessory protein
mRNA	Messenger ribonucleic acid
PD	Panic disorder
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus
R160W	Arginine160Tryptophan
R163Q	Arginine163Glutamine
Rev	Reverse
SAM	Sympathetic-adrenal medullary
SCN	Supra-chiasmatic Nuclei
SERT	Serotonin reuptake transporter
SNP	Single Nucleotide Polymorphism
Tris	Tris(hydroxymethyl)aminomethane
TPH	Tryptophan hydroxylase
V60L	Valine60Leucine
V66M	Valine66Methionine
V92M	Valine92Methionine

Abstract

Major depressive disorder (MDD) and depressive states cause a major toll on individuals suffering from it and on society as a whole. Today's therapeutic options for MDD are far from ideal and a majority of patients experience relapse. Current therapeutic strategy suffers from the lack of biological cues of individual variability in disease etiology. The steroid "stress hormone" cortisol has received particular focus in this context, due to its interaction with the neurotransmitter serotonin, which has been strongly associated with mood control, cognitive and emotional processes. Assessing and understanding cortisol dynamics in a clinical setting, especially in terms of its diurnal rhythm, will increase understanding of subtypes of MDD. The increase in cortisol production in response to awakening (the cortisol awakening response, or CAR) is frequently disturbed in MDD, and is the focus of a long-term ongoing study of cognitive therapy strategies at the University of Oslo, Department of Psychology. Sample preparation, hormone measurements, and genotyping for molecular-genetic factors underlying individual variation is performed in a collaborative project including the research group in physiology and neurobiology. While the main study is still ongoing, the primary goal of this thesis is to use preliminary data to determine if the study design and procedures are expedient to 1) Reveal overall CAR dynamics as well as individual trait variation in the study population 2) Reveal the influence of genetic polymorphisms in the serotonin system known to affect cortisol production and its diurnal rhythm. In addition, prompted by recent findings in a comparative (teleost) model, I aimed to conduct a preliminary study to assess the presence and distribution of single nucleotide (SNP) polymorphisms in the melanocortin system in contrast groups of high and low cortisol producing individuals. I found that a clear CAR was observed in the study population indicating that the chosen methods (micro radioimmunoassay) and sampling procedures were viable. In addition, there was a near significant gender effect towards increased CAR in females. Different genotypes affecting serotonin transporter expression also showed a significant effect on CAR magnitude. Lastly, when sequencing study participants selected according to high- or low cortisol production status, four different SNPs were discovered in the melanocortin 1 receptor, two of which were exclusively present in the low producing cortisol group. Based on these findings, functional studies are needed to further explore the role of MC1R in stress-reactivity. Additionally a larger sample size and a control group, which have been already added in the ongoing project, will help elucidate the possible interactions between gender and genotype on CAR in remitted patients.

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1 Introduction:

1.1 MDD: a major toll on human welfare

According to the World Health Organization, major depressive disorder (MDD) is the leading cause of disability worldwide and affects approximately 10% of the human population worldwide. MDD is a heterogeneous mental disorder that inflicts an array of symptoms, including decreased life quality, loss of energy, inability to experience pleasure, sleep disturbances, and can lead to suicide in severe cases (Heshmati and Russo, 2015, de Bruin et al., 2016). In MDD, cognitive and emotional biases in attention direct perception and memory towards negative input. The associated cognitive, emotional, and behavioural changes hinder individuals to maintain personal relations, their ability to endure daily tasks, and to properly function as a part of society.

There is a high degree of individual variation in predisposition for depression, aetiology and treatment outcome (Hodgkinson et al., 1987, Milaneschi et al., 2016), However, research on subtypes of depressive states (e.g. in atypical depression and melancholia) and their underlying genetic factors has revealed a number of associated risk factors (McGuffin et al., 1996, Gutierrez et al., 1998, Gold and Chrousos, 2002, Aklillu et al., 2009). In effect, the biological substrates most commonly implicated in MDD include the brain serotonergic signalling system and the endocrine stress response, which in pathological states decreases neural plasticity and confers a range of physiological and behavioural alterations (Mattson et al., 2004, Pittenger and Duman, 2008, Ruhe et al., 2015). The onset of depression is clearly associated with exposure to acutely traumatic or chronic intermittent stress in a number of studies (Hammen, 2005, Gutierrez et al., 2015), but the psychoneuroendocrine profile is highly dependent on the individual (Hohne et al., 2014). Identification of biological and genetic markers for stress responsiveness and sensitivity to the inhibiting effects of negative experiences has been identified as a key research area to improve therapeutic outcome for the treatment and prevention of MDD. To accomplish this, increased insight into molecular-genetic links between stress - and neurobiological function is pivotal, as these fundamental functions are often disturbed in affective disorders. Following, I will review the knowledge base in this field and outline the context for the project of which this thesis is a part.

1.2 Stress, cortisol, serotonin and MDD

As noted above, there is a strong link between depression and stressful life events. Individuals exposed to chronically stressful circumstances (e.g. childhood abuse, parental neglect, lack of control and predictability) or acute traumatic events are over-represented in MDD (Checkley, 1996, Hammen, 2005). Furthermore, physiological and neurobiological responses to stress are deeply involved in emotional control (Paykel, 2003, Hammen, 2005, Herbert, 2013). In particular, the onset and course of MDD has been reported to be associated with disturbances in cortisol (a steroid hormone) and serotonin (5-Hydroxytryptamine, 5-HT, a monoamine neurotransmitter) signalling (Carroll et al., 1968, Carroll, 1982, Portella et al., 2005, Aguilera et al., 2009). Implication of an altered serotonergic signalling system in development and maintenance of depressive states is widely accepted (Heninger et al., 1984, Graeff et al., 1996, Mahar et al., 2014). In fact, alleviation of depressive symptoms can be seen with uptake of selective serotonin reuptake inhibitors (SSRIs) targeting serotonin transporters, and it is believed that SSRIs exerts its antidepressant effect by increasing the availability of synaptic 5-HT (Delgado et al., 1988, Blasey et al., 2011, Le Noury et al., 2016). However, the traditional view that low levels of 5-HT are causative or implicated in MDD is being challenged by post-mortem and animal studies (for review see Andrews et al., 2015). Alternatively, the "high 5-HT hypothesis" claiming that 5-HT transmission is elevated in multiple depressive phenotypes has gained wider acceptance (Barton et al., 2008, Andrews et al., 2015). Serotonergic neurotransmission and hypothalamic-anterior-pituitary (HPA)-axis activity co-regulate each other (Dinan, 1996, Hoglund et al., 2002, Goel et al., 2014, Kurhe et al., 2015). Serotonergic innervation has been shown to directly facilitate or inhibit HPA axis activity (Lowry, 2002), and modulates HPA-axis activity through projections that innervate the amygdala, hippocampus, and anterior hypothalamus (Hensler, 2006). 5-HT has a generally stimulating effect on corticotropin-releasing factor (CRFF), adrenocorticotropic hormone (ACTH) and cortisol release (Lesch et al., 1990, Dinan, 1996,) and this effect has been found to be significantly enhanced in some depressive states (Maes et al., 1991). In addition, the serotonergic system has been shown to exert postnatal "programming" of developing HPA-axis architecture, resulting in long-term effects in neural plasticity associated with increased susceptibility to stress-related diseases (Andrews and Matthews, 2004). In fact, trophic / structural effects of 5-HT (i.e. stimulating neurogenesis and other aspects of neural plasticity) has been the major focus for research on 5-HT in depression,

rather than immediate signalling effects on post-synaptic neuronal activity (see references below).

Moreover, HPA-axis activity expressed as hypercortisolaemia, decreased diurnal rhythm, elevation in basal glucocorticoid (GC) levels and/or elevation in evening GC levels, is associated with the onset and continuation of psychiatric disorders, including depressive states (Halbreich et al., 1985, Gillespie and Nemeroff, 2005, Bremmer et al., 2007, Jarcho et al., 2013). The exact mechanisms underlying a shift from adaptive stress responses to pathogenesis remains ambiguous, but deteriorating physiological and psychological effects caused by elevated cortisol are well documented and conserved between animals and humans (Masters et al., 1989, Tombaugh et al., 1992, Gubba et al., 2000, Lupien et al., 2009, Sorensen et al., 2013).

Notably, cortisol and 5-HT are deeply involved in regulating brain structural processes (neurogenesis, synaptogenesis, and other aspects of neural plasticity) necessary for normal cognitive and emotional function (Mattson et al., 2004, Pittenger and Duman, 2008), and therefore it is pivotal to study and understand how both the 5-HT and cortisol systems may influence core features of MDD (e.g. decreased emotional regulation, cognitive function and lethargic behaviour) (Drevets, 2001, Hasler et al., 2004, Krishnan and Nestler, 2008, Pittenger and Duman, 2008), which will be reviewed below.

1.3 Cortisol and 5-HT play central roles in neural plasticity and MDD

Neural plasticity and molecular processes such as long-term potentiation (LTP) are necessary for both emotional control and cognitive processing of events such as formation and retrieval of episodic memories (Duman, 2002, Duman, 2004, Pittenger and Duman, 2008, Duman et al., 2016). When these fundamental processes are impeded, difficulty in emotional regulation and subjective unpredictability increases while ability to form appropriate adaptive responses in future-related settings decreases. The neurofunctional link between neural and structural plasticity and emotional regulation is a core feature in psychological disorders and pivotal in MDD development and treatment (Phillips et al., 2003, Price and Drevets, 2010). 5-HT and cortisol have opposing effects on neural plasticity in that 5-HT stimulates neural plasticity, while the effect of cortisol on neural plasticity is dependent on exposure and duration (Bou-Flores et al., 2000, Bonnin et al., 2007, Sale et al., 2008, Daubert and Condron, 2010, Jonassen and Landro, 2014, Radley et al., 2015). Transient cortisol exposure increases neural

plasticity, while on the other hand chronic cortisol exposure hinders it (McEwen, 1998, McEwen, 2004, Kavushansky et al., 2006, Roozendaal et al., 2009).

Another link between cortisol and 5-HT signalling is the fact that chronic treatment with SSRIs tends to decrease basal cortisol levels and the cortisol awakening response (CAR) in MDD patients (Warner-Schmidt and Duman, 2006, Ruhe et al., 2015). Furthermore SSRIs increase expression of brain-derived neurotrophic factor (BDNF) (Chen et al., 2001, Karege et al., 2005, Warner-Schmidt and Duman, 2006, Heldt et al., 2007, Lee and Kim, 2010). BDNF is a neurotrophic peptide critical for neural plasticity (e.g. axonal growth, neuronal survival and synaptic plasticity), as well as survival and morphological differentiation of 5-HT neurons (Lu, 2003, Mattson et al., 2004). Reciprocally, main trophic effects of 5-HT are mediated by stimulatory effects on BDNF (Homberg et al., 2014). Notably, in the SSRI treatment there is a preliminary period of therapeutic delay lasting up to four weeks in which cortisol levels are transiently increased concurring with a worsening of symptoms (Lahti and Barsuhn, 1980, Meltzer and Maes, 1994, Malberg et al., 2000, Haslam et al., 2004). This is believed to be mediated by alterations of signalling pathways caused by increased cortisol levels (Bale, 2006, Murgatroyd et al., 2009, Albert and Benkelfat, 2013, Booij et al., 2013). Some of the alterations induced by elevated cortisol leads to reduced neural plasticity (Sorensen et al., 2013, Pittenger and Duman, 2008). Acute stress has been shown to reduce hippocampal BDNF mRNA expression, which is likely achieved through the concurrent effect of stress on the serotonergic signalling system, which exerts regulatory control over BDNF (Smith et al., 1995, Duman and Monteggia, 2006). In summary, impaired neural plasticity in MDD and the normalization of this process associated with remission makes the molecular-genetic background of the neuroendocrine and endocrine systems modulating neural plasticity interesting targets in studying MDD.

1.4 Predisposing genetic factors and individual disease histories

Why do some individuals become depressed and others not?

The answer to why stress-related diseases only affects some people, but not others who have similar life experiences, lies within the most complex biological system; the brain. Life history and biological factors (genetic traits, epigenetic makeup, alterations of histones, as well as noncoding RNA) governs the ability an individual has to cope with its environment. Arguments that MDD vulnerability is genetically influenced are seen in the fact that

depression is heritable, being the case in about 40% of incidents (Sullivan et al., 2000, Fernandez-Pujals et al., 2015). However, molecular-genetic investigations have for decades been limited by relatively few highly penetrant vulnerability genes that are identified in MDD. Still, there are several genetic traits identified as risk factors or even causal factors to the etiology of MDD. These risk loci include the *serotonin reuptake transporter* (SERT) and the serotonin transporter linked polymorphic region (5-HTTLPR), the *tryptophan hydroxylase* (TPH), the BDNF gene V66M single nucleotide polymorphism (SNP), and epigenetic modulations of the GC receptor promoter region (Lesch et al., 1996, Caspi et al., 2003, Zhou et al., 2005, Gizatullin et al., 2006, Aguilera et al., 2009, McGowan et al., 2009, Gutierrez et al., 2015). All these genes and regulatory sequences code for central proteins involved in the serotonergic signalling system, neural plasticity and/or the stress activated HPA-axis.

1.5 Activation and regulation of the HPA– axis

Stress and circadian rhythms

Cortisol is a steroid hormone synthesized in and secreted from the adrenal cortex in response to stress, diurnal rhythms or hypoglycemia. Koolhaas et al., (2011) suggests that the term "stress" should be restricted to conditions where demands exceeds the natural regulatory capacity of an organism, and in particular uncontrollable and unpredictable conditions. In humans stressful events activate two physiological responses: the immediately reacting sympathetic-adrenal medullary (SAM) system, resulting in the release of epinephrine and norepinephrine; and the relatively slower activation of the HPA-axis (figure 1). Cortisol and its equivalent corticosterone in many mammalian species, contributes to regaining homeostasis when facing stressors. Cortisol acts by suppressing energy-demanding non-essential functions and reallocates resources towards vital functions such as increasing attention, arousal, gluconeogenesis and regulating the heart and circulatory system (Chrousos and Gold, 1992, Sapolsky et al., 2000). Prior to HPA- axis activation, fibers from the amygdala convey information about stress to the hypothalamus, which in turn induces the synthesis of CRF and arginine vasopressin (AVP) in neurons of the paraventricular nucleus. CRF and AVP are released into the hypophysal portal system where CRF (and to a lesser degree AVP) binds to CRF1 receptors located on the anterior pituitary, signalling to corticotrophic cells to transcribe and process POMC (Chrousos et al., 1985, Lamberts et al., 1984 Chrousos, 1995). POMC is a precursor peptide that is cleaved post-transcriptionally

into different hormones, including melanocortin stimulating hormones (MSH) and adrenocorticotropic hormone (ACTH). ACTH is released into the vascular system and reaches the steroid-producing zona fasciculata within the adrenal gland and binds to the membrane bound melanocortin receptor (MC2R), which results in the activation of adenyl cyclase and subsequent increase in cyclic-adenosine monophosphate (cAMP) (Neves et al., 2002, Gantz and Fong, 2003, Roy et al., 2012). cAMP in turn stimulates the activity of steroidogenic enzymes such as cytochrome p450 family 11 subfamily B member 1 (CYP11B1), which converts progesterone into cortisol and helps catalyse the formation of adrenal steroid hormones (Simpson, 1979, Pallan et al., 2015). Following this is the release of cortisol along with another important class of steroid hormones, the mineralocorticoids (MC), collectively known as corticosteroids, into the vascular system. Cortisol enters cells via passive diffusion and binds to glucocorticoid (GC) receptors. Cytosolic GC receptors with bound ligand translocate into the nucleus and act as both transcription factors and modulate protein complexes affecting transcription of many genes (Kirschbaum et al., 1996). Cortisol mediates negative feedback on HPA-axis activity at the level of the paraventricular thalamus, hippocampus, hypothalamus, pituitary, and the prefrontal cortex ensuring that cortisol levels return to baseline levels and stress responses are terminated when stress is overcome (Jaferi and Bhatnagar, 2006, Furay et al., 2008, Evanson et al., 2010, Russell et al., 2010, Radley and Sawchenko, 2011, Hill et al., 2011). This fact becomes clear in the cortisol awakening response (CAR), a rapid increase and decrease of cortisol in response to awakening. And the distinguishable CAR is often utilized in non-invasive monitoring of cortisol dynamics in humans (Fries et al., 2009).

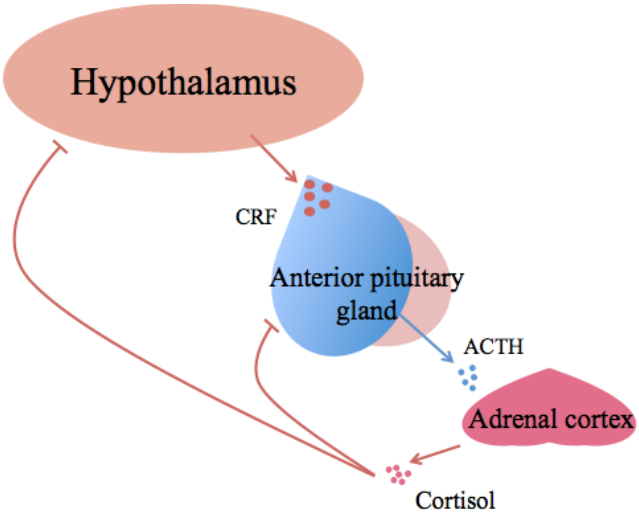

Figure 1: **The HPA- axis**. In response to stress CRF-synthesizing cells of the paraventricular nucleus of the hypothalamus release CRF into the hypophysal portal system. CRF acts on the anterior pituitary gland stimulating release of ACTH. ACTH reaches the adrenal glands through the vascular system and induces the synthesis and release of corticosteroids. (Illustration private)

1.5.1 The Cortisol Awakening Response

In non-stressful situations, night cortisol levels are low, but rise in the early morning hours peaking approximately 20-40 minutes after awakening, in a process known as the cortisol awakening response, or CAR (Pruessner et al., 1997, Wust et al., 2000b, Clow et al., 2010). After peaking cortisol levels decline gradually throughout the day, although other factors such as perceived stressors overrule this circadian rhythm (figure 2). The circadian diurnal rhythm of the HPA-axis and CAR is mainly under the control of the supra-chiasmatic nucleus (SCN) pacemaker in the hypothalamus (Clow et al., 2010, Postnova et al., 2013). The SCN receives neural input from the retina and other brain modalities (e.g. the intergeniculate leaflet, dorsal raphe nucleus and the median raphe nucleus). Circadian cues are communicated from the SCN to other organs including the adrenal glands via humoral and neural pathways (Moore and Eichler, 1972, Engeland and Arnhold, 2005, Dibner et al., 2010). In the process of awakening neural innervation from the SCN increases HPA-axis activity and adrenal sensitivity (Dijkstra et al., 1996, Buijs et al., 1997, Buijs et al., 1999, Buijs et al., 2003). The awakening process causes hippocampal activity to decrease, and as a result hippocampal inhibition on the SCN ceases, hence allowing CRH levels to increase leading to a rise of ACTH and cortisol (Postnova et al., 2013, Fries et al., 2009). In healthy individuals the CAR is mainly driven by ACTH (Ebrecht et al., 2000). However, ACTH is not solely responsible, since adrenal denervation causes loss of circadian cortisol secretion (Jasper and Engeland, 1994, Lilley et al., 2012). It is also worth noting that the SCN and adrenal glands harbour intrinsic molecular feedback loops that are necessary for maintaining normal diurnal circadian rhythm (Oster et al., 2006).

Abnormal CAR is implicated in a variety of psychosocial processes and health conditions including MDD, remitted patients and individuals at high risk for developing MDD (e.g. those with various risk loci and/or familial history) (Sephton et al., 2000, Pruessner et al., 2003b, Clow et al., 2004, Steptoe et al., 2004, Nater et al., 2008, Vreeburg et al., 2010). CAR in remitted MDD patients has been shown to be elevated compared to healthy controls (Bhagwagar et al., 2003, Vreeburg et al., 2009, Vreeburg et al., 2010). However a portion of individuals do not have the expected cortisol increase after awakening, Vreeburg et al., (2009) found that almost 30% of previously depressed patients did not have any response. Ambulatory studies could suffer from inaccuracy in sampling, however when carefully monitoring awakening time and sampling compliance 15% of individuals had no cortisol increases post awakening (Dockray et al., 2008). It is emerging that the nature of the CAR

and the relatively simple way to measure it makes it a better-fit option than other HPA-axis measurements (e.g. the dexamethasone suppression test) to detect HPA-axis disturbances (Pruessner et al., 1999, Cowen, 2010). Previous studies have reported that both attenuated and increased CAR is associated with depression, however this disparity could be due to differences in severity or how studies have defined a depressive state (Huber et al., 2006, Vreeburg et al., 2010, Hardeveld et al., 2014). Another explanation to this is that different endophenotypes of depression have been associated with different CAR, for instance mild depression is associated with similar CAR as healthy controls, remitted patients and patients with moderate depression is associated with increased CAR, and severe depression is associated with decreased CAR (Bhagwagar et al., 2003, Veen et al., 2011). Moreover antidepressant use has shown to normalize HPA-axis disturbances and CAR in remitted patients (Ruhe et al., 2015).

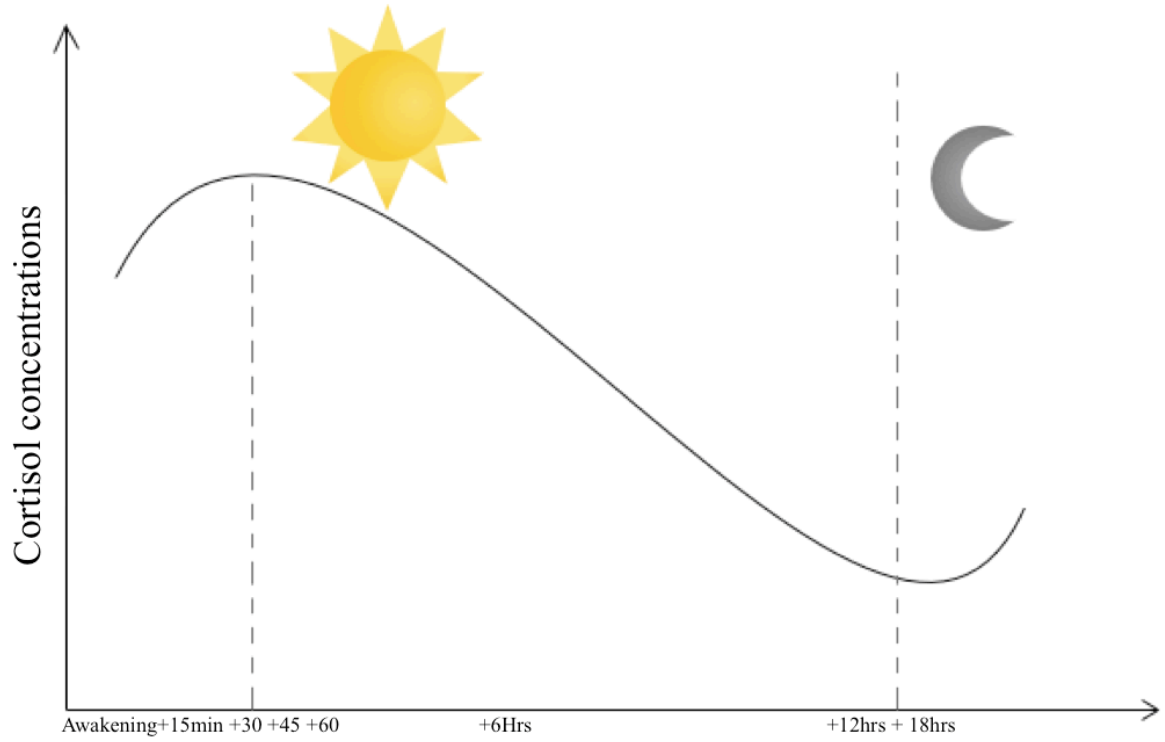

Figure 2. CAR. Adapted by Elder et al., (2016)

1.6 The cortisol awakening response and cognitive therapy: an ongoing study

This thesis utilises data gathered in the ongoing project "Secondary prevention of depression applying an experimental Attention Bias Modification procedure". The project is lead by Professor Nils-Inge Landrø and his research team at the Department of Psychology at the University of Oslo. The aim of the main project is secondary prevention of depression in previously depressed patients, by modification of a negative attention bias through the computerized Attention bias modification (ABM) procedure seen in relation to candidate genes for serotonin transportation/reuptake. The main project is ongoing, and will consist of results from approximately 400 participants when completed. The inclusion criteria include a history of recurring depressive episodes. The participants should be in remission at the time of the experiment. As stated in the previous section disturbances of CAR do not always normalize during remission, but normalization of CAR is seen in some who take antidepressants (Ruhe et al., 2013). While the main study is still ongoing, the main goal of my thesis is to use preliminary data to validate study design and procedures used. This study will assess whether this particular population display a CAR, and if so the CAR magnitude and distribution. If these results are promising, the next goal is to assess whether genetic polymorphisms affect cortisol production and diurnal rhythm.

Measuring CAR from saliva cortisol

95% of cortisol in blood is either bound to cortisol binding globulin (CBG) or to albumin with lower affinity. Unbound cortisol enters saliva and reflects free circulating cortisol levels (Vining et al., 1983, Mendel et al., 1989, Perogamvros et al., 2012). For the sake of studying hormones in humans, saliva extraction has long been a preferred method (Vining et al., 1983, Gozansky et al., 2005, Cardoso et al., 2009). This sampling method is relatively non-invasive, low cost, with high compliance and can easily be done in an ambulatory setting. Studies consistently report high correlations between serum and salivary cortisol, indicating that salivary cortisol levels reliably estimate serum cortisol levels (Rantonen et al., 2000, Poll et al., 2007, Trifonova et al., 2013, Mezzullo et al., 2016). The greatest challenges with ambulatory studies are ensuring high compliance to protocols to maximize identical sample treatment. Individual differences in mental function (e.g. depressive state) have been shown to affect compliance (DiMatteo et al., 2000). Low adherence to sampling protocols such as delaying the awakening sample can flatten the peak (relative to actual awakening cortisol

levels) and thus mimic a deficiency (Kudielka et al., 2003, Dockray et al., 2008). However most studies report that most study participants accurately follow sampling protocols, and sampling compliance inaccuracy is predominantly a problem in elderly subjects (Kraemer et al., 2006, DeSantis et al., 2010).

1.7 Serotonin-transporter-linked polymorphic region genotype influence on CAR

Genetic polymorphisms in the serotonergic signalling system have been implicated in MDD and stress vulnerability (Gutierrez et al., 1998, Caspi et al., 2003, Gutierrez et al., 2015). In particular the 5-HTTLPR affecting expression of the 5-HT transporter and thus availability of synaptic 5-HT has contributed to the monoamine hypothesis of MDD. A 43 bp insertion/deletion polymorphism in the promoter region of the serotonin reuptake transporter influences the transcription rate of the serotonin transporter (*5-HTT*) gene, the short (*s*) allele being transcriptionally less efficient than the long (*l*) allele. The short low-expressing variant of the 5-HTTLPR leads to fewer reuptake transporters present in the presynaptic terminal thus elevated 5-HT concentration, and leads to increased stress and depression vulnerability (figure 3) (Caspi et al., 2003, Karg et al., 2011, Miller et al., 2013, Andrews et al., 2015). In addition, there is a single nucleotide polymorphism (SNP) rs25531 (A/G) that can be found in the context of both *s* and *l* alleles and effects transcriptional activity of *5-HTT* (Hu et al., 2005). Previous studies have shown that the 5-HTTLPR genotype has influence on CAR and HPA-axis reactivity (Wust et al., 2009, Frokjaer et al., 2013). Most studies show that *ss* carriers have a higher CAR peak (Chen et al., 2009, Goodyer et al., 2009, Frokjaer et al., 2013). However; Wust et al., (2009) found that in males genotype *ll* was associated with increased CAR, while in females genotype *ss* was associated with the highest CAR. Moreover *ss* carriers produce a higher and prolonged cortisol response following standardized stress-tests (Jabbi et al., 2007, Gotlib et al., 2008).

The goal of the main project, which this thesis is a part of is to examine whether there are genotype associated differences in effect from attention bias modification training (ABMT) ("cognitive therapy"). However this thesis has analysed only a preliminary subset of samples from the ongoing longitudinal study, so a primary objective of this thesis has therefore been to assess the feasibility of the study design. To this end I endeavered to investigate whether an effect of the 5-HTTLPR genotype on CAR magnitude and HPA-axis reactivity is detectable in the currently available data-set.

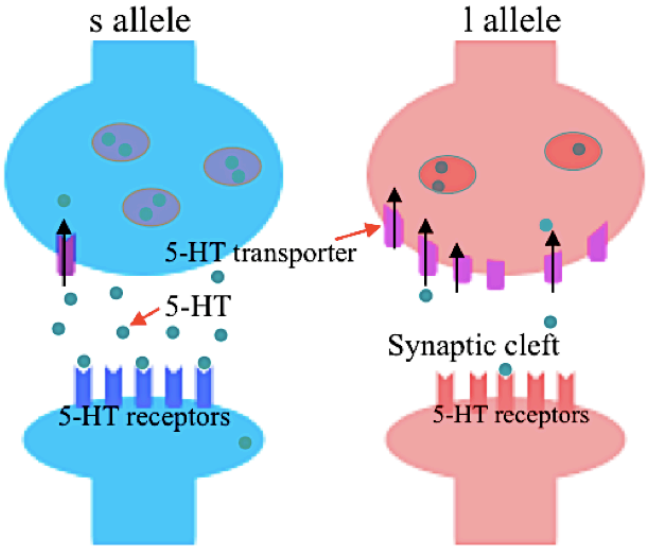

Figure 3. 5-HTTLPR polymorphism. The *s* allele results in less 5-HT transporters present in the presynaptic end terminal and reduced reuptake capacity of synaptic 5-HT. The *l* allele results in more 5-HT transporters in the presynaptic end terminal thus more efficiently terminating 5-HT signalling from the synaptic cleft. (Illustration private)

1.8 Towards novel candidate genes: Polymorphisms in the melanocortin 1 receptor modulates stress reactivity in model systems

In addition to examining CAR dynamics and the possible influence of the 5-HTTLPR genotype, prompted by recent findings in a comparative (teleost) model (Khan et al., 2016), I aimed to conduct a preliminary study to assess the presence and distribution of SNP polymorphisms in the melanocortin system in contrast groups of high and low cortisol-producing individuals. The role of the melanocortin system in the stress response and diurnal rhythms is to integrate signals from the pituitary in the adrenal gland (described in section 1.5). The melanocortin system involves five distinct G protein coupled melanocortin receptors 1 -5 (MC1-5R), and Melanocortin 2 receptor accessory protein (MRAP). MRAP regulates trafficking and function of MC2R and is required for its signalling, in contrast MRAP reduces signalling from the four other receptors (Cooray and Clark, 2011). ACTH binds MC2R, which stimulates steroidogenic cells to synthesize and release cortisol (Neves et al., 2002, Gantz and Fong, 2003, Roy et al., 2012). MC1R is involved in pigmentation, increased melanoma risk, nociceptive tolerance and intriguingly, polymorphic variations of it have previously been implicated in MDD, bipolar disorder and antidepressant response (Wu et al., 2011, Hayden and Nurnberger, 2006, Cheng et al., 2006). A newly discovered polymorphism in the melanocortin system that alters interrenal sensitivity to ACTH and thus

post stress production of cortisol in teleosts (Khan et al., 2016), has to the best of my knowledge never been studied previously in individuals remitted from MDD. Albeit a limited material is available presently, an attempt to investigate possible associations between CAR magnitude and MC1R polymorphisms was therefore included in this thesis.

The melanocortin system is highly conserved between humans and teleosts so it is conceivable that shared genotypes coexist as well (Ringholm et al., 2002). Khan et al., (2016) examined the presence of possible polymorphic candidate genes in the melanocortin system of rainbow trout involved in stress sensitivity (HPA-axis reactivity). This approach was feasible since heritable variation of post-stress cortisol production in the trout model seems to be determined by sensitivity to ACTH in steroid producing tissues, rather than variation in hypothalamic output (Pottinger and Pickering, 2001). In these teleost fish a SNP variation L176M in *MC1R* causes altered MC2R function resulting in distinct stress reactivity. The postulated mechanism of increased reactivity in HR fish was improved affinity for MRAP in MC1R with the L176M SNP, and hence less availability for the MRAP interaction necessary for MC2R trafficking and signalling.

Interestingly, the human *MC1R* gene is highly polymorphic with 348 known SNPs according to the National Center for Biotechnology Information (NCBI, November 2016). Using protein-protein BLAST protein sequence alignment, the corresponding position of the Rainbow trout *MC1R* SNP in the human *MC1R* gene was identified as amino acid (AA) position 170, and there was 60% shared sequence identity. No publications for SNPs in this position in humans were found, but NCBI stated two known human SNPs (rs770551931 and rs778831502) that were validated by frequency or genotype data reported with hitherto unknown significance. Furthermore, MDD patients with hypercortisolaemia have been shown to have normal plasma ACTH and cerebrospinal fluid CRH concentrations, suggesting that sensitivity at the adrenal level is responsible for dissociation between ACTH and cortisol in these patients (Wong et al., 2000, Stetler and Miller, 2011). In these patients, altered function of the MC2R or factors that directly influence steroidogenesis could potentiate HPA – axis reactivity and the CAR (Nussdorfer, 1996, Ehrhart-Bornstein et al., 1998).

Although a far reach, I wished to investigate whether there were variations in MC1R that were associated with CAR magnitude. A promising approach to tackle the challenge of revealing usually small effects of gene variants on complex phenotypes is to assess mechanistically meaningful endophenotypes (Hasler et al., 2004). To do this, ideally a standardized stress test (e.g psychosocial stressor testing) should be employed to directly measure stress responses in addition to responses to a natural stressor (awakening), but there

were limitations in experiments possible to execute in this context. Based on previous studies implicating CAR as a indicator of HPA-axis reactivity (Schmidt-Reinwald et al., 1999, Wust et al., 2000b, Gotlib et al., 2008, Chen et al., 2009), I concluded that using partial CAR as an index of adrenocortical responsiveness to stress was viable in attempt to identify high and low cortisol producing subjects, and then analyze whether MC1R SNP frequency distributions were unequal between these contrast groups.

Aims and hypothesis of Study

- The main goal of this thesis is to use preliminary data from an ongoing study to determine if the study design and procedures are expedient to:

1) Reveal overall CAR dynamics as well as individual trait variation in the study population.

H1) Individuals with depression have been associated with different CAR dynamics. Therefore, I expect to find individual differences in a diverse group of remitted individuals as well.

2) Reveal the influence of genetic polymorphisms in the serotonin system known to affect cortisol production and diurnal rhythm.

H2) Serotonin transporter expression has been shown to affect CAR in healthy and depressed patients, and different genotypes will influence CAR magnitude in remitted patients as well.

- The subordinate goal of this thesis is to assess the presence and distribution of single nucleotide (SNP) polymorphisms in the melanocortin system in contrast groups of high and low cortisol producing individuals.

H3) SNPs in the melanocortin receptor can alter ligand and/or accessory protein binding and influence stress sensitivity.

2 Materials and methods

2.1 Saliva cortisol

2.1.1 Study design

Included in this thesis is data from 146 candidates enrolled in the project at an early stage. Study participants were given information by trained personnel employed at the Department of Psychology to complete saliva sampling at home. The study was approved by the regional ethical committee. The sampling procedure was designed by collaborators at the Department of Psychology and would upon completion of the project be executed 5 times, distributed over a year as follows: initial sampling (start up), and after; 2 weeks, 1, 6, and 12 months of initiation (timeline figure 4). Currently, very few patients have undergone the complete sampling and treatment program. Therefore, in this thesis I have used data from the first day of sampling to assess inter-individual variation in CAR and underlying genetic mechanisms, while drawing on data from the second sampling day (two weeks after study initiation) and correlational analysis to confirm trait stability in individual CAR magnitude. Longitudinal dynamics in CAR, treatment effects, and possible interactions between genetic factors and treatment outcome will be analysed by the project consortium when all data become available.

'CAR' is usually defined as the dynamic of post-awakening cortisol secretion and can be measured as the area under the curve (AUC) with respect to ground (AUC_G) or with respect to the increase (AUC_I), which requires a minimum of 3 morning sampling time points (Pruessner et al., 2003a, Stalder et al., 2016). However in this study only 34 patients with a complete curve (5 morning samples) were available, for reasons of project design that were beyond my influence. The first enrolled candidates were instructed to take 3 samples at 3 different time points, one in the evening, and two the subsequent morning. Specifically, in sequence the first saliva sample was taken the evening preceding ABMT initiation between 20:00 and 22:00 hours, thereafter two samples were obtained the following morning, the first immediately after awakening and the second 15 minutes later. Of the original 146 study participants 22 study participants were excluded due to missing samples. 124 study participants completed sampling at start up. However the sampling program was redesigned so that in addition to the evening sample, candidates took 5 consecutive samples in the morning within 15 min intervals, thus measuring an extended CAR. 34 candidates in the present data set completed the later sampling regime. This change was implemented in order

to further assess CAR dynamics, as cortisol has been reported to reach its peak after 30-45 minutes after awakening. These data were later used to assess whether the 15 min sampling point was indicative of that individual's cortisol level at later time points (c.f. results section, 3 figure 7) when the actual peak occurred. Therefore, in the following the second morning cortisol sample (awakening + 15 min) was used as a predictor of the morning peak, while average cortisol levels was used to assign individuals into contrast groups of high and low cortisol producers.

Figure 4: Timeline of sampling. ABMT included in timeline although this was not focused on in the present study. Half of the study participants had the active ABMT condition. The remainder went through sham training. Each sampling time-point includes 3 (or 6) samples, one in the evening and two or five in the morning immediately after awakening and with 15 minute intervals. We have focused on the first two sampling time-points indicated by red arrows. (Illustration private)

Sampling procedures:

Saliva samples were obtained using the Sarstedt Cortisol Salivette® Device; polypropylene/ low density polyethylene tubes with a separate internal detachable compartment containing a cotton swab. One hour before sampling participants were instructed not use any types of tobacco, eat, brush their teeth, or drink alcohol. At sampling time participants kept the cotton swab in their mouth until saturation with saliva. In addition to the simple protocol that accompanied the take-home sampling devices, trained personnel employed at the Department of Psychology explained the protocol to each participant verbally. Samples were delivered by the patients themselves to the Department of Psychology and stored in freezers at -18 °C, until all samples were transferred to -80 °C freezers within weeks. The samples were thawed on ice and cold centrifuged at 4 °C, 1000 * rcf for 15 minutes. The saliva samples were then transferred into 1,5mL Eppendorf tubes and stored at -80 °C. Samples were brought to Radbound University in Nijmegen on dry ice for radio immunoassay analysis.

2.2 Radioimmunoassay

The protocol used for cortisol RIA in micro plates was refined from previous methodology as described by (Gorissen et al., 2012). In short 3-5 96-wells Micro-Assay-Plates (Greiner-Bio-one: 655094; White/µClear - high-binding) were prepared each day (see appendix section 7

for details). Wells were prepared by adding cortisol antibody (Abcam: ab1949; Cortisol Antibody[xm210] monoclonal and IgG purified) diluted in coating buffer into all wells, except A-specifics that receive coating buffer only. Plates are incubated overnight at 4°C. Following incubation wells are washed with 200 µl wash buffer and then with 200 µl of block buffer. Plates were then placed in a heat cabinet at 37°C for 1-hour incubation. Blocking buffer is removed from the wells by decanting and immediately thereafter 10µl standards (Sigma: H4001-5G; Hydrocortisone ≥98% HPLC), Saliva samples are thawed on ice for ~1-2 hours. 10 µl saliva samples and controls (assay buffer) are added in duplicates except controls, which are added in triplicate. Finally 90µl 3H-Cortisol tracer (PerkinElmer: #NET396250UC - Hydrocortisone (Corstiol,[1,2,6,7-3H(N)]-),[1,2,6,7-3H(N)]- 250µCi (9.25MBq) is added into each well and left to cold incubate over night at 4°C. thereafter incubation plates are washed three times with wash buffer. Prior to β-measurement scintillation solution is added to all the plates. Values that are obtained are directly translated into saliva cortisol concentration. Samples were assayed in duplicate ($r = 0.8849$). The intra-assay coefficients of variation for our low and high quality control standards were 4.3 and 6.7%, respectively. The inter- and intra-assay variation coefficients are 12.5 and 2.5% respectively. Cross-reactivity of the antibody with cortisone was < 1%.

2.3 DNA extraction, purification, Polymerase Chain Reaction (PCR)

The 5-HTTLPR genotyping, which will be utilized in a parallel study, was analysed by the Psychopharmacological Department at Diakonhjemmet Hospital. The DNA isolation procedure was mainly compiled by my fellow student Hallvard Heiberg (unpublished).

2.3.1 DNA extraction and purification using Qiagen or Isohelix kits

To obtain DNA from buccal epithelium cells study participants were instructed to rub an Isohelix SK-1S DNA Buccal Swab for 1 minute on the inner cheek. DNA samples were stored in room temperature until analysis. DNA isolated was carried out using kits: DNeasy Blood and Tissue from Qiagen or BFK-50 from Isohelix. The following procedure description is freely available for the BFK-50 Isohelix kit. In short 20µl PK solution was added to the tube containing the buccal swap, after a 30-minute incubation the entire sample was transferred into a 1.5ml tube and 400µl BP solution was added. Samples were then centrifuged at maximum speed (13.4K rpm/12,000 x g) for 10 minutes. The resulting pellet

contained both the DNA and other impurities. Supernatant was carefully removed. 50-150µl TE solution was added to the pellet and vortexed to resuspend pellet in the solution. After 2 – 5 minutes the DNA is fully hydrated and to remove the undissolved impurities the tube is re-spun for another 15 minutes at maximum speed. Lastly the supernatant containing the DNA was transferred to a sterile 1.5 ml tube and stored in a -20° C freezer until amplification.

2.3.2 Polymerase chain reaction (PCR) amplification MC1R

Human sequences for MC1R were retrieved from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/gene/) and primers were designed by Professor Dag Inge Våge and myself using the Primer3plus program (http://primer3plus.com). Primers were synthesized by ThermoScientific (see table 1 for primer sequences and accession numbers).

2.3.3 MC1R PCR amplification

For primers MC1R pair 1 and 2 DyNAzyme EXT DNA Polymerase kit from Thermo Scientific was used. See table 1a – c for the PCR reaction composition and PCR conditions, executed on PCR machine Eppendorf Mastercycler® gradient. To confirm amplification of desired PCR fragments the PCR product was stained with GelRed Nucleic Acid Stain (Biotium, Inc.) and run on a 1.5 % SeaKem LE Agarose gel (Lonza) and compared to Thermo Scientific GeneRuler 50 bp DNA Ladder.

a)

Gene name:		Product size (bp)	Primer sequence 5´→ 3´	Accession number
MC1R	Primer pair 1	600	Fwd: ACCTGGAGGGGAAGAACTGT Rev: CGTGCTGAAGACGACACTG	NM_002386.3
MC1R	Primer pair 2:	599	Fwd: GTGGACCGCTACATCTCCAT Rev: CCAGACCACACAATATCACCA	NM_002386.3

b)

Component	Volume (µl)
H_2O Milli-Q	18,15
10x PCR Buffer -Mg	2,5
50 mM $MgCl_2$	0,75
10 mM dNTPs	0,5
Taq DNA Polymerase	0,1
Forward primer	1
Reverse primer	1
Template DNA	1
Total volume	25

c)

Cycle step	Temp.	Time	Cycles
1st denaturation	94 °C	3 min	1
Denaturation	94 °C	45 s	30
Annealing	60°C	30 s	
Extension	72 °C	50 s	
Final extension	72 °C	10 min	1
	4 °C	Hold	

Table 1: a) primer pairs used in PCR. b) and c) PCR reaction mixture and cycling conditions used for MC1R.

High through-put sequencing

Prior to sequencing, PCR products were cleaned-up using USB® ExoSAP-IT® PCR Product Cleanup. In brief, 5 µl of post-PCR reaction product is mixed with 2 µl Exo-SAP-IT reagent. The mix is incubated at 37°C for 15 minutes degrading remaining primers and nucleotides. Incubated further at 80°C for 15 minutes to inactivate ExoSAP-IT reagent. The resulting PCR products were subsequently sequenced directly by GATC Biotech AG (Cologne, Germany), using an ABI 3730xl DNA Analyzer systems (96 capillary instrument; LIGHTrun sequencing) with a read error probability of 1:100. Each PCR product was sequenced with forward and reverse primers separately, minimizing chances of read errors. Sequences were aligned and screened for SNPs using the programs phred, phrap and consed (Ewing and Green, 1998, Gordon et al., 1998).

2.3.4 Genotyping 5-HTTLPR

The 5-HTTLPR polymorphism was genotyped by the Psychopharmacological Department at Diakonhjemmet Hospital essentially as described in detail elsewhere (Gelernter et al., 1997, Stein et al., 2006). In brief a real-time fluorescence Light Cycler instrument was used to amplify genomic DNA by polymerase chain reaction (PCR) in a final volume of 20 µL using Light Cycler Faststart DNA SYBR Green kit (Roche cat no. 12239264001) with specific primers (0.5 µM) (Gelernter et al., 1997) generating a long 419 bp or a short 375 bp PCR product. Cycle conditions were initiated by 10 min denaturation (95 °C) followed by 45 cycles at 95 °C (10 s), 66 °C (10 s) and 72 °C (10 s).

2.4 Data analysis

As noted above, ideally 3 or more sampling time-points are required to calculate total cortisol secretion output by using the formula for AUC_G or AUC_I as described by Pruessner et al., (2003). However due to the low number of candidates who had enough samples for these calculations (n = 34), I decided to instead focus on individual sampling time-points, and average CAR. These data were highly variable, with an evening range from 0.05 to 12.9 ng/ml, and morning ranges from 0.1 to 37 ng/ml. Initial tests focused on simple parameters such as CAR presence and magnitude (i.e. comparing evening and morning samples) and gender effects. The data did not meet requirements for parametric analysis, homogeneity of variance was for instance not possible to obtain between evening and morning samples or between males and females even after extensive transformations (assumption tests, Statistica

software). This ruled out the use of parametric statistics and more advanced multivariate methods, e.g. principal component analysis for the identification of contrast groups, or stepwise model building for interactions between gender, genotype, and sample time. Hence, non-parametric Kruskal-Wallis ANOVA and Dunns Multiple Comparision test followed by Bonferroni correction was used to analyse differences between sampling time-points. Possible gender effects were tested by means of repeated Mann-Whitney U-tests, and correlations between individual values at different sampling points was investigated by means of Spearman`s rank correlation.

This analysis ascertained that the 2nd morning sample point (awakening + 15 min) was representative for expected peak values (measured in only a subset of the study subjects, see Results section 3.1 below), and also that this indicator of CAR magnitude correlated strongly to the measurement obtained two weeks later. Gender differences were not significant, hence cortisol values at this time point were pooled between males and females. Thus negating the factor that variability was higher in females than males, parametric criteria were achieved after log transformation and removal of data points now appearing as extreme outliers (indicated in figure 9, Grubb's test). Anova GLM with number of *s*-alleles as a single continuous predictor (regressor) variable was used to analyze effects of the 5-HTTLPR polymorphism. For the purpose of identifying possible effects of MC1R gene polymorphisms, although sequencing of all study participants would be informative, it was possible to only include a subset of all samples due to time limitations. Contrast groups of individuals with the highest observable morning cortisol levels (high cortisol producers, HCP, n=20) vs. lowest cortisol levels (low cortisol producers, LCP, n=20) were identified by ranking the average of the two first cortisol measurements after awakening (after Wust et al., 2000b).

3 Results

3.1 Cortisol data

3.1.1 Complete CAR in a subset previously depressed patients

Figure 5 shows salivary cortisol concentration at 6 different sampling points in the part of the study population that underwent the extended sampling protocol. Kruskal-Wallis ANOVA revealed a highly significant effect of sampling time ($p<0.001$) with expected diurnal rhythmicity, i.e. cortisol production at the evening time point was significantly reduced compared to all morning samples. (Dunns Multiple Comparision test followed by Bonferroni correction, $p <0.001$). Numerically, the highest values tended to be seen at the third morning sampling point, (i.e. awakening + 30 minutes), but the apparent increase in cortisol production after awakening never reached statistical significance, due to a high degree of individual variability between participants (Dunn's $p > 0.05$ all morning vs. morning comparisons).

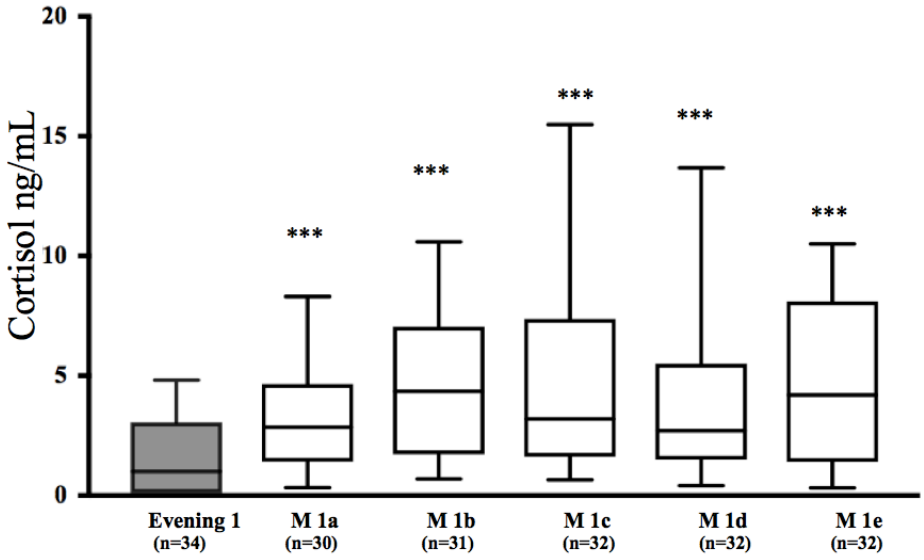

Figure 5: CAR at start up: (presented as median, 1st and 3rd quartile box plot, 10-90 % of minimum and maximum) Cortisol concentration (ng/mL) on Y-axis, time points on X-axis. Evening 1 is the start-up sampling time-point taken in the evening between 20:00-22:00 hours, M1a: morning 1a, cortisol sampling immediately after awakening, M1b: morning 1b, sample taken 15 minutes after M1a. M1c: morning 1c sample taken 15 minutes after M1b. M1d: morning 1d sample taken 15 minutes after M1c. M1e: morning 1e sample taken 15 minutes after M1d.

3.1.2 Sex differences and CAR in previously depressed patients

First, comparisons were performed at each time point to check for possible gender effects, which indicated a trend towards higher cortisol production in females during both the evening, awakening sample and 15 minute post awakening sample (figure 6a-c). After Bonferroni correction for multiple testing a near significant difference (initial p = 0.052) at the 2nd sampling point were discarded (corrected p=0.14), so in the following analysis data from males and females are pooled (c.f. figure 10-11).

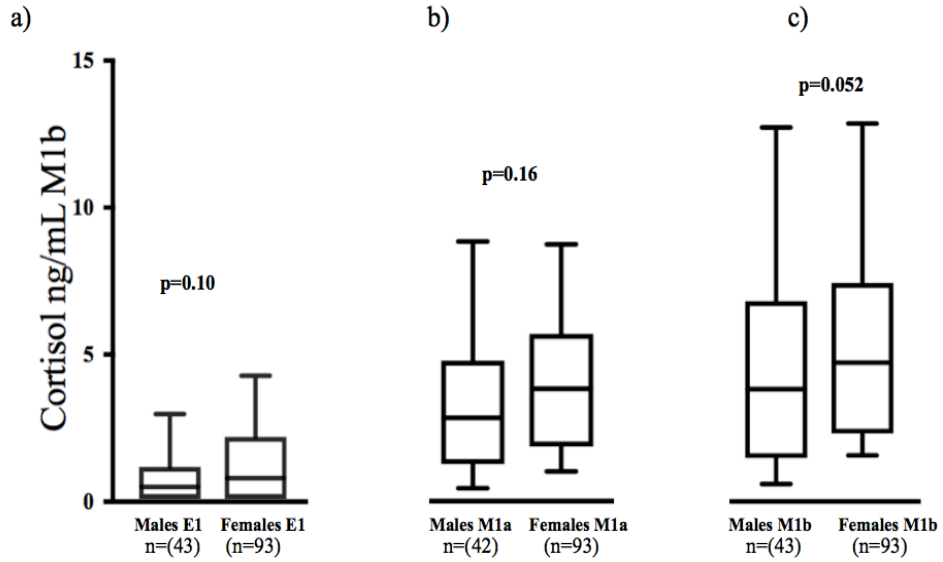

Figure 6. Sex differences in CAR. (presented as median, 1st and 3rd quartile box plot, 10-90 % of minimum and maximum). a) Salivary cortisol at evening 1 (E1), b) Morning sample 1 or awakening (M1a) c) morning 1b (M1b) second morning sample or 15 min post-awakening (c) in males and females. NS.

In males and females gender effects were near significant (p=0.0523, Mann-Whitney U-test) at the 15 minutes post awakening sampling point (figure 6c). Data were significantly more variable in females at the evening sampling point (Bartlett's assumption test, p<0.001), and were not normally distributed at any time point (Kolmogorov-Smirnov test). Also in the evening data there was a trend towards higher cortisol in females than in males (Mann-Whitney U-test, p = 0.096).

Even though there was a high level of inter-individual variability in cortisol production, there was a significant positive correlation between both morning sampling cortisol values (figure 7). This indicates that morning cortisol production is determined by neuroendocrine processes that are at least generally consistent over the 15 minute time scale employed in this sampling regime.

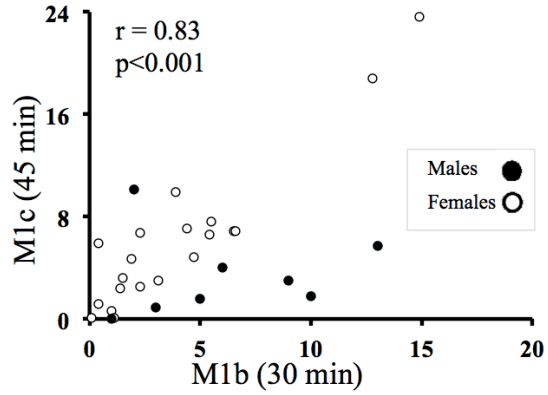

Figure 7: Correlation between 2nd and 3rd sampling point in the n= 34 data subset with additional sampling points after 15 min. No significant differences between males and females were seen at either axes, Spearman rho and p values are shown.

Thus, in the following analysis of possible genetic contribution to explain inter-individual variability I decided to focus on the 2nd morning sampling point as the assumed best predictor of overall individual cortisol production.

3.1.3 Individual variability and consistency

From the (n = 130) individuals who had sampled both morning samples (immediately after awakening and 15 minutes post-awakening), 25,4 % (n = 33) had no increase or negative cortisol response after awakening, 28,5 % (n = 37) had a cortisol awakening response of less than 50%, 46% (n=60) had a awakening response of more than 50%. To investigate whether cortisol at different sampling points were related, I checked if evening cortisol predicted morning cortisol at awakening. Spearmank correlation analysis showed a clear positive correlation between evening and morning cortisol values (figure 8a). Notably, there was also a strong positive correlation between morning cortisol values of the first day and 2 weeks later (figure 8b).

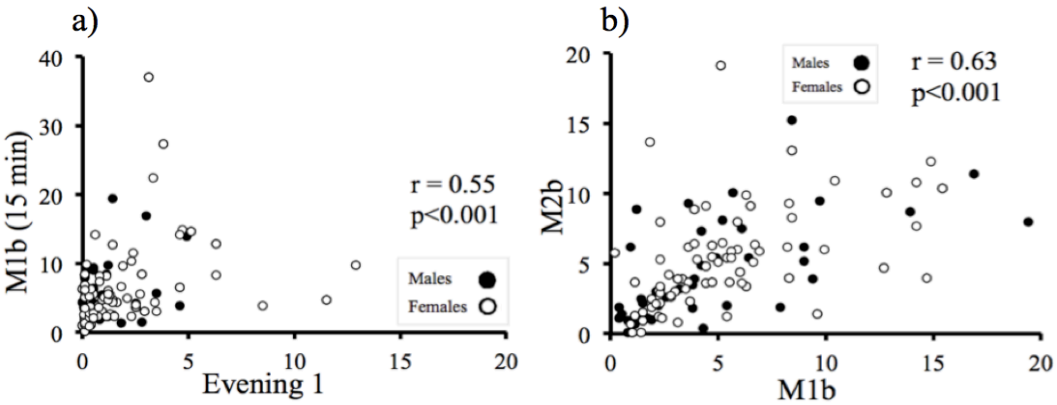

Figure 8: a) Correlation between evening and morning cortisol (2nd morning sampling point) in the full data set. No significant differences between males and females were seen at either axes, Spearman rank correlation r and p values are shown **b):** Correlation between morning cortisol (2nd morning sampling point) at day 1 and 14 of the study. No significant differences between males and females were seen at either axes, Spearman rank rho and p values are shown in each panel.

3.1.4 HCL LCP

The average mean of cortisol levels in the HCP and LCP groups were 11,2 ng/mL (±SD 5,3) and 1,1 ng/mL (±SD 0,4) respectively.

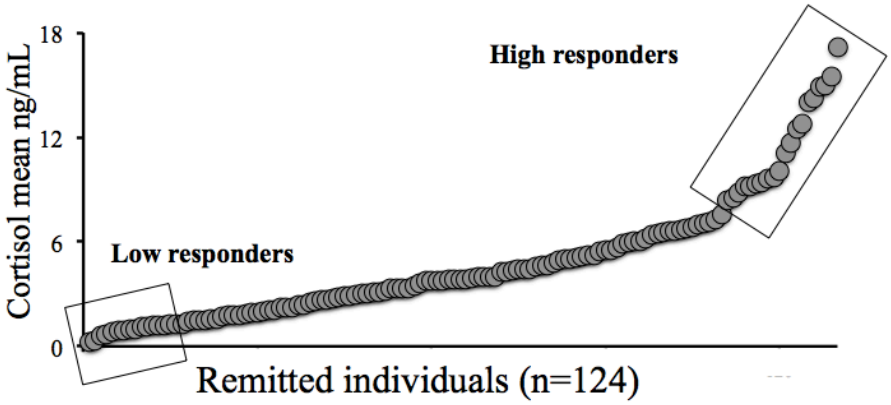

Figure 9: scatter plot with mean cortisol morning values from awakening sample and 15 minutes post awakening. 20 High responders and 20 low responders shown in boxes.

3.1.5 5-HTTLPR polymorphism

In pooled data for morning cortisol (awakening + 15 min), thus negating the fact that variability was not homogenous between females and males, parametric criteria were achieved after after log transformation and removal of 4 extreme outliers (Grubb's test). A general linear model ANOVA with number of *s*-alleles as a single continuous predictor (regressor) variable indicated a significant effect of the 5-HTTLPR polymorphism, in that saliva cortisol values tended to decreased as a function of *s*-allele number (*ll-sl-ss*, ANOVA statistics $F_{(1,124)} = 6.1$, $p = 0.01$). At later time points fewer samples were available and differences were not significant, but numerically *ss* allele carriers appeared to increase and reach a peak at a later time point than *ll* and heterozygous *sl* genotypes (figure 11).

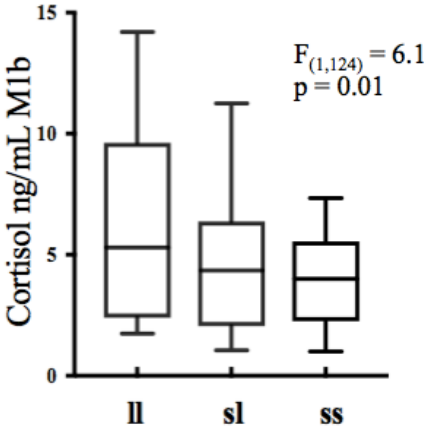

Figure 10. Mean cortisol concentrations at post awakening +15 minutes in entire data-set sorted according to genotype. (presented as median, 1st and 3rd quartile box plot, 10-90 % of minimum and maximum). F and p values are shown.

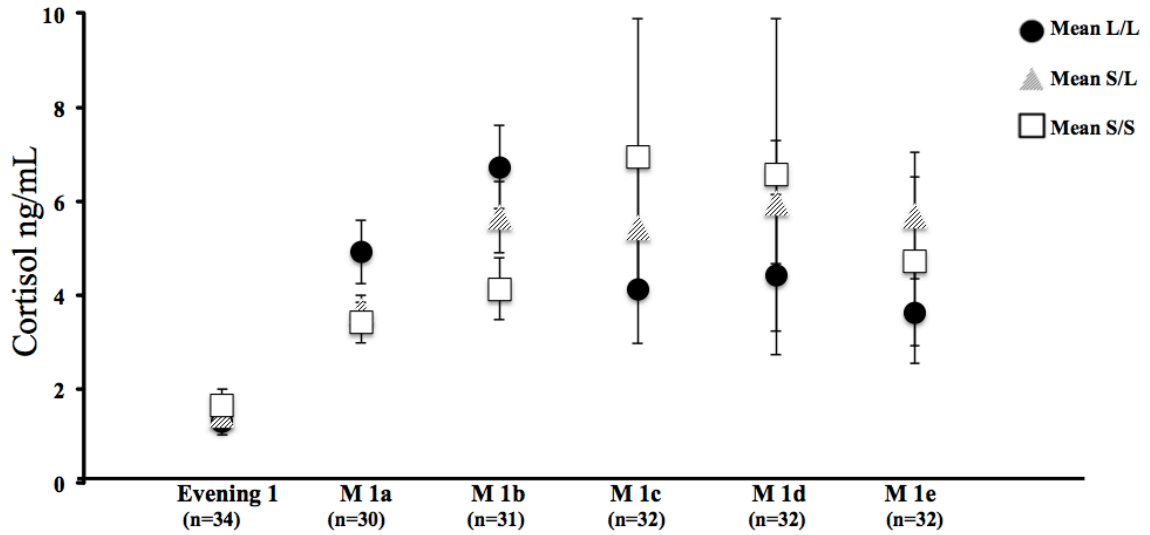

Figure 11: CAR in study population with extended CAR measurement sorted according to genotype. After 30 minutes (i.e. M1c) *ss* carriers have reached a higher peak than other genotypes. Mean cortisol values shown ±SEM. NS.

3.2 MC1R gene variation

Unfortunately there was some difficulty amplifying genes from a few of the candidates, and due to time constraints I decided to go forward with sequencing of the available material. I achieved amplification of MC1R genes from 12 HCP and 13 LCP individuals. Four SNPs (V69L, V92M, R160W and R163Q) were ascertained in the MC1R sequences. All these SNPs have previously been reported in the Natural Variants database, a catalog of known human G-protein coupled receptor (GPCR) polymorphisms (Kazius et al., 2008, Isberg et al., 2016, Munk et al., 2016). The frequencies of SNPs in the contrast groups were generally much higher (or lower) in respect to the general population frequency (see table 2). See table 2 for frequency distributions of SNPs in general population vs HCP and LCP groups. Interestingly, the SNPs exclusively found in the LCP group (V60L and V92M) are both located in intracellular loop I (Ringholm et al., 2004). Despite the low available n, one homozygous and (V92M) and three heterozygous individuals (V60L: 2, V92M: 1) corresponds to 5 mutated alleles of 24 possible, while this frequency was 0 of 26 in the HCP contrast group. The difference in frequency distribution is significant (p=0.02, Fisher's exact test), suggesting that the intracellular loop (IL) I mutations may in fact be functionally linked to cortisol production in a way opposite to the AA160-163 mutations in IL II, of which a majority tended to occur in the HCP phenotype (6/26 vs 1/24, p=0.10). These latter mutations are close to the polymorphism reported by Khan et al., (2016) which would be in AA position 170 in the human genome (see figure 12 for two dimensional model of MC1R).

Phenotype	Reference id	Nucleotide substitution	HCP:LCP	WT (n)	Allele frequency in overall population*	Allele frequency in remitted individuals	SNP mut. frequency in LCP and HCP individuals	LCP Het. (n)	LCP Hom. (n)	HCP Het. (n)	HCP Hom. (n)
V60L	rs1805005	G/T	9:9	16	G=0.8846 T=0.1154	G=0.941 T=0.059	LCP=0.125 HCP=0.0	2	0	0	0
V92M	rs2228479	G/A	9:9	16	G=0.8846 A=0.1154	G=0.91 A=0.09	LCP=0.2 HCP=0.0	1	1	0	0
R160W	rs1805008	C/T	10:9	16	C=0.9286 T=0.0714	C=0.886 T=0.114	LCP=0.047 HCP=0.214	1	0	2	1
R163Q	rs885479	G/A	10:9	17	G=0.9231 A=0.0769	G=0.945 A=0.055	LCP=0.0 HCP=0.125	0	0	2	0

Table 2: Table with MC1R SNPs frequency in general population vs frequency in remitted HCP and LCP individuals. WT: wildtype or most commonly occurring SNP. Heterozygous: Het, Homozygous: Hom..*frequency in European population derived from NCBI database, 1000 Genomes Project recourses (November 2016).

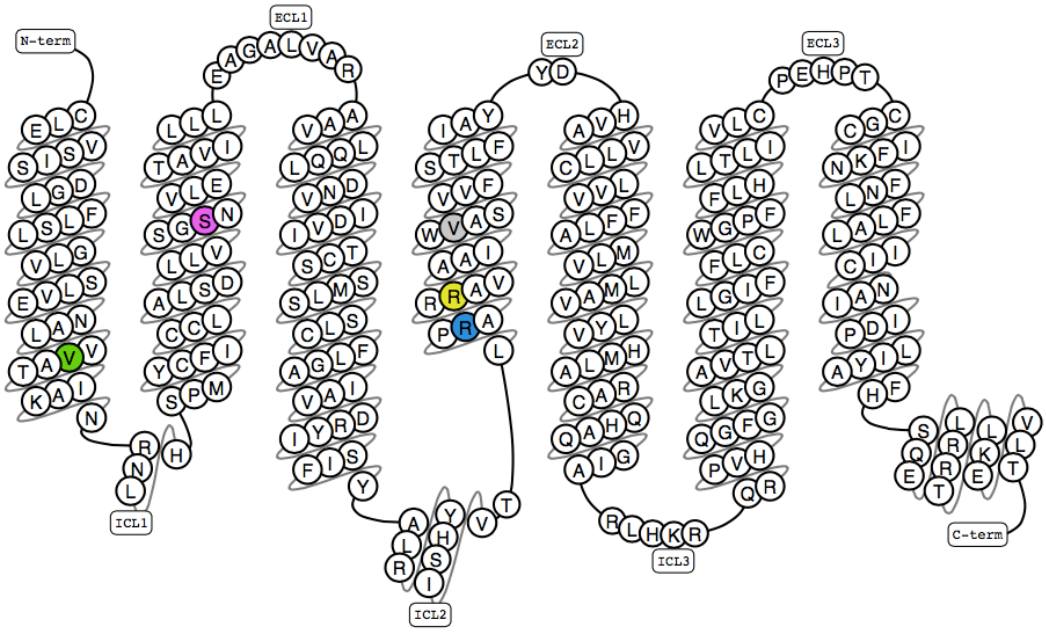

Figure 12: Two-dimensional model of human MC1 receptor (Accession number Q01726). Model generated from GPCR database website (www.gpscdb.org). Amino acids are shown in circles. Colored residues indicate positions for SNPs found in study participants. Green; V60L, pink; V92M, blue; R160W, yellow; R163Q, not found in population AA 170 grey; 170V.

4 Discussion

The current study was part of a large longitudinal study lead by the Department of Psychology at UiO. The primary aim of the main project is secondary prevention of depression in previously depressed patients, by modification of negative attention bias through the computerized Attention Bias Modification (ABM) procedure. Treatment outcome is also seen in relation to the 5-HTTLPR genotype.

The experimental design was largely pre-determined before the collaboration with the Department of Biosciences was established. However the genetic material of all study participants was available and permission to investigate the presence of genetic components possibly altering cortisol dynamics and neural plasticity granted. The chief focus of this thesis serves as a validation of methods and procedures, as the self-sampling protocol may lead to some bias or inconsistencies (see section 1.6). To this end, I analysed cortisol concentrations in saliva samples currently available to confirm the presence and dynamics of the CAR in the study population. Cortisol diurnal variations were generally as expected in healthy population with a clear increase in morning vs. evening samples. Additionally the 5-HTTLPR genotype affected morning cortisol levels 15 min post awakening. However, the direction of this effect was in contrast to a majority of other studies, but not completely without precedence (see section 4.2 below for further details). Furthermore, CAR magnitude was found to be longitudinally stable during over a two week period, strengthening the assumption that cortisol production can be seen as a stable "personality" trait at least partly influenced by genetic factors (Wust et al., 2000a, Bartels et al., 2003b, Federenko et al., 2004). As for the influence of the MC1R gene, four common SNPs were found, none previously known to alter stress sensitivity. In contrast groups no individuals had the Met170Val SNP corresponding to SNPs found to alter stress sensitivity in rainbow trout model systems. Although two of the SNPs were found close to the AA170 position, molecular kinetics and functional properties of the MC1R protein are not sufficiently characterized to determine whether any of these SNPs could modulate cortisol production by the MRAP interaction suggested by Khan et al., (2016).

4.1 Complete CAR in previously depressed patients

The majority of study population only provided measurements from the 15 first minutes of CAR referred to as partial CAR, while a subset of 34 individuals measured extended CAR over an hour after awakening, including the apparent cortisol peak and decline. A significant increase in cortisol between evening and morning was shown in the entire dataset, but there was no significant difference between any of the morning time-points. The cortisol peak during CAR is typically not reached until 30-45 minutes post awakening (Wust et al., 2000b). In line with CAR literature, data from the individuals who had delivered 5 samples in the morning showed a trend that cortisol continued to increase after 15 minutes.

Morning sample 15 minutes post awakening indicates subsequent peak

In the subset with extended CAR measurements the 15-minute awakening sample was found to correlate strongly with the subsequent cortisol values. From this observation I count upon that using the 15 minutes post-awakening sample available for the majority of study subjects as an indicator of CAR magnitude is relevant. Preferably extended CAR measurements would be used to determine CAR dynamics, and most studies on CAR have typically measured extended CAR (Wust et al., 2000b, Steptoe et al., 2010, Frokjaer et al., 2013, Stalder et al., 2016). Studies that measure only two time points emphasize that results must be interpreted with some caution as the cortisol peak may in some patients occur later or earlier (Wust et al., 2000b).

Intradividual variability

In the complete data set there was a median saliva cortisol increase of 40% from the awakening moment to 15 minutes after. A total of n=33 of all study participants (n=126) had no increase or decreases between the first and second sample. This observation could be partly due to lack of sampling compliance or mislabelling, but the frequency of sampling or labelling errors is not known. Other studies have also found a similar portion of study population to be non-responsive, meaning that they do not have a cortisol surge in response to awakening (Wust et al., 2000b, Vreeburg et al., 2009). Furthermore in studies that carefully monitor sampling and awakening time 15 % of individuals are non-responders (Dockray et al., 2008). In line with this the majority of study participants displayed increases during the first 15 minutes. Previous studies have shown that disturbed CAR does not normalize during remission (Bhagwagar et al., 2003, Bhagwagar et al., 2005, Aubry et al.,

2010, Vreeburg et al., 2010). In agreement with our results studies have shown that high-risk individuals, remitted individuals and different subtypes of depression are associated with attenuated, normal and increased CAR (Huber et al., 2006, Vreeburg et al., 2010, Hardeveld et al., 2014). Inclusion criteria in this study was that participants must be remitted from MDD, therefor CAR during a depressive episode was not assessed. Whether observed CAR in the present study populatiuon was influenced by remitted status can not be ascertained at present. However a control group has been added to the study retrospectively, but this was not included in the original design, and thus not in the data subset available to me. Other variables such as the effect of SSRI medication would be of interest to investigate when the whole dataset becomes available and, conceivably, more advanced multivariate models requiring higher degrees of freedom could be applied.

Sex differences

In this study there was a strong trend for an increased cortisol response in females 15 minutes after awakening (figure 6a,c) ($p=0.0523$ prior to correction for multiple comparisons). Gender differences in magnitude and time course of CAR are widely accepted (Pruessner et al., 1997, Jabbi et al., 2007, Wust et al., 2009). When the full data set with extended CAR becomes available this trend may become clearer, especially since females have a cortisol peak that occurs slightly after males (Wust et al., 2000b). Gender differences in cortisol levels and prevalence of MDD are not observed in prepubertal children or postmenopausal women and have lead to the hypothesis that ovarian hormones contribute to this gender difference (Kessler, 2003, Steiner et al., 2003, Netherton et al., 2004). Higher oestrogen levels have a positive action on cortisol and CBG levels resulting in higher cortisol levels in females, possibly contributing to the higher prevalence of MDD in females as well (Feldman et al., 1979, Netherton et al., 2004, White et al., 2006, Ter Horst et al., 2009). Despite not finding a significant effect of gender in this dataset there is every reason to more closely investigate possible gender effects on the genetic versus environmental contribution to CAR variability when the complete dataset becomes available. Additionally females displayed a significantly higher variability in evening cortisol levels (figure 6 a, assumption test). This could potentially indicate increased subjective stress perception during the day in females relative to males (Chaplin et al., 2008).

Interindividual stability

In line with literature on CAR individual stability of CAR magnitude was observed over a limited time period suggesting a genetic influence (figure 8). This view is supported by twin studies showing heritability of HPA-axis reactivity (Wust et al., 2000a, Bartels et al., 2003a, Federenko et al., 2004). The observed stability also argues that the methods and procedures used are consistent enough to override variability incurred by the sampling, handling and analytical procedures (time in room temperature, sampling compliance etc.). Notably, in addition to the correlation between evening and awakening values, there was also a strong positive correlation between morning cortisol values of the first day and 2 weeks later (figure 8b). In summary the data suggest that cortisol production and CAR is a consistent individual trait, which may have a genetic background.

Sampling procedure and method

A main objective of this study was to evaluate preliminary data to determine suitability of chosen methods. Sampling procedure was explained to the study population in advance, and was conducted by a group of diverse candidates in respect to age, education, mood, emotional state, cognitive faculties and compliance to testing procedure may have been variable. Several study participants failed to soak the cotton swabs with saliva and only a small amount of fluid was obtained (sometimes not enough for duplicate measurements) or no fluid at all. One participant did not complete sampling because the cotton swab induced an involuntary retching reflex. Sampling compliance to protocol was not tested in this study, and is not commonly done in large studies. However in large studies it is generally recommended to test a selection of the study population using instruments that aid in objective measuring awakening time and sampling time (Stalder et al., 2016). Obtaining accurate sampling information can reduce bias on CAR estimates through data exclusion strategies and statistical modeling approaches (Stalder et al., 2016). Although our primary interest was cortisol, cotton materials are poorly suited when cortisol metabolites or other saliva substances are of interest since they can alter pH and is an absorbent material (Shirtcliff et al., 2001, Papacosta and Nassis, 2011). However for this purpose cotton-based sampling approaches such as Salivettes® are commonly employed, although initially employing a passive drool technique would eliminate the above mentioned factors (failing to soak, or involuntary retching reflex) as well as man labour, as cotton swabs need to be centrifuged after sampling (described in materials and methods). Time and ambient temperature between sampling and freezing could be very variable, depending on time of day when patients were

admitted to ABMT training. However relatively stable CAR measurements over time and expected CAR dynamics argues that the methods are consistent enough to override variability incurred by the sampling and analytical procedures. Thus, in conclusion possible confounding issues were apparently not detrimental to the study.

4.2 5-HTTLPR

In line with most literature studies we found a trend that the 5-HTTLPR genotype had an influence on CAR magnitude (figure 10) (Wust et al., 2009, Frokjaer et al., 2013). However in contrast to some findings (Chen et al., 2009, Frokjaer et al., 2013) and in agreement with others (Wust et al., 2009), we found that *ll* carriers had a higher cortisol response than *ss* carriers and that *ls* carriers were intermediate cortisol responders (Gotlib et al., 2008). Similarly a study by Jabbi et al., (2007) found that high-risk individuals that were *ll* homozygous had slightly higher baseline cortisol levels, but they also that female *ss* carriers responded stronger to stress and exceeded l carriers and males. Regrettably we were not able to obtain information about the A/G SNP, the l_G genotypes transcriptional activity being similar to the *s* allele. However in a study by Wust et al., (2009) the inclusion of the A/G SNP and 5-HTTLPR genotypes in sorting healthy individuals according to expression rates of 5-HT transporters did not modify results significantly. Wust et al., (2009) found that males with *ll* genotype had the largest CAR, but in females the highest CAR was seen in *ss* genotype. One possible interpretation of the present results is the possibility that *ss* carriers show lower CAR magnitude only shortly after awakening, with a delayed peak. As stated earlier *ss* carriers are at more risk for future depressive episodes, but on the other hand the *s*-genotype has also been suggested to be a plasticity gene so *ll* carriers could be burdened by lower plasticity and experience more severe symptoms (Fox et al., 2008). This may have affected accuracy in sampling compliance, and may persist even in remission (DiMatteo et al., 2000).

In conclusion, the current study shows that 5-HTTLPR genotype affects CAR magnitude, but precise dynamics description requires an extended number of subjects following the complete sampling procedure with 5 morning sample timepoints.

4.3 MC1R sequences

Although we found no SNP in AA position 170 of MC1R, corresponding to the L176M SNP governing post-stress cortisol production in teleost, other SNP's showed a possible effect on CAR. Hence, potential effect on stress reactivity in humans is not ruled out. Due to experimental limitations regarding human study subjects the selection regime could not be done based on response to standardised stress tests. However previous studies have shown that CAR and HPA-axis reactivity is positively correlated (Gotlib et al., 2008, Chen et al., 2009). We found four different natural occurring SNPs that in part distinguished the HCP and LCP groups. As seen in figure 12 all of these SNP´s appear in transmembrane segments. Functional studies of SNPs in transmembrane regions have shown to affect the ability to respond to the natural ligands (Ringholm et al., 2004). SNPs V92M and R163Q are associated with remission after desipramine treatment and with depression respectively (Wu et al., 2011). The V60L genotype MC1R results in poor cell surface expression and subsequent decreased basal activity (e.g. cAMP signaling) (Schioth et al., 1999, Beaumont et al., 2005, Conn and Ulloa-Aguirre, 2010) The V92M mutant binds α-MSH with 100-fold lower affinity compared to wild-type thus also resulting in lower cAMP signalling (Ringholm et al., 2004). The R160W genotype leads to decreased cAMP production, and is strongly associated with red hair and fair skin (Herraiz et al., 2009). The R163Q genotype displays normal ligand stimulated cAMP signaling, but a decrease in MAPK activation which is attributed to the position of the SNP known to interact with G protein (Doyle et al., 2012). Interestingly in the LCP group we exclusively found V60L (two individuals heterozygous) and V92M (one individual hetero- and the one homozygous), these genotypes are both associated with impaired cAMP production in response to MSH binding. In the HCP group R160W and R163Q were almost exclusively present, apart from one individual from the LCP group who was heterozygous for the R160W SNP (table 2), notably this individual in the LCP group was the individual with the highest cortisol mean within the group. Due to the close proximate positions of R160W and R163Q it is not unthinkable that these two genotypes could both interact with G-protein binding. G proteins are signal transducers that regulate a range of functions (Neves et al., 2002), some of which could potentially be implicated in stress reactivity. Further study of protein-protein interactions are needed to determine whether or not these two SNPs can be assigned similar effects in causing decreased HPA-axis reactivity through the interaction with MRAP suggested by Khan et al (2016). Moving forward it would have been interesting to implement a stress test and base

the selection regime on stress responses as well as CAR thus further validating whether CAR is indicative of HPA-axis reactivity and assessing whether different genotypes are correlated with CAR dynamics and/or stress/HPA-Axis reactivity.

Comorbidites

The inclusion criterion in the study was a history of recurring depressive episodes, but no present depression. In this study 70 (50,7%) of study participants had comorbidities while 68 (49,3 %) only had a MDD diagnosis (data from 8 individuals not available). These comorbidities complicate the biological understanding of MDD, for instance substance abusers; alcoholics in particular, have shown higher cortisol levels (Badrick et al., 2008). MDD is rarely a diagnosis that comes alone, generalized anxiety disorder, substance abuse, social phobia, panic disorder, hypomanic symptoms, agoraphobia, obsessive-compulsive disorder are commonly present and were represented within the study population as well. Comorbidities are associated with distinct features, for instance blunted adrenocortical reactivity in spite of a normal CAR has been reported in those with panic disorder (PD) (Petrowski et al., 2010). However in disagreement to this finding, there were three individuals with PD comorbidity in both the HCP group and the LCP group. Those with PD in the HCP group had a robust CAR, but since we did not expose participants to any stress paradigm we cannot conclude that for these individuals CAR was not an index of a hyper reactive adrenocortical axis.

5 Conclusion and future perspectives

The methods and procedures of measuring CAR in remitted MDD individuals was successful based on the presence of a distinct CAR in the majority of individuals. Additionally the 5-HTTLPR genotype showed a trend towards *l* genotypes having increased CAR during the first 15 minutes of the CAR. Although the latter result is in contrast to most findings, I also found a strong trend towards females having a higher CAR magnitude, which is in agreement with other studies. There were large interindividual differences in remitted patients as well in agreement with other studies. Furthermore promising preliminary data on MC1R polymorphisms in contrast groups was observed as well. The consolidation of these findings argue that chosen methods and procedures are expedient to measure CAR and detect interindividual variations and molecular-genetic correlates of it.

Research on genetic-molecular features of MDD can benefit from distinguishing between meaningful endophenotypes. To further reach this goal extended demographic knowledge as well as symptom presentment could help ameliorate the background of CAR dynamics. For instance stressful life events, number of depressive episodes and duration, chronic pain, drug use, smoking and drinking habits, ethnicity and of course information about perceived stress at the measuring time-point were lacking in this study and would help assess CAR variability and underlying causative mechanism in the future. In addition the inclusion of a healthy control group will further strengthen the study. For a broader assessment of the HPA-axis reactivity to different kinds of stress, a stress test could be included. A dexamethasone / CRF test could indicate if negative feedback is impaired or if abnormalities in the pituitary or adrenal responses are involved, potentially giving insight into what therapeutic strategies could benefit individual patients. For instance in this study project all study participants undergo a brain MR scanning, a procedure that could potentially evoke stress in some participants and could serve as a "stress test". Importantly MC1Rs direct role on stress reactivity depends on co-expression of MC1R and MC2R in steroid producing tissues, as is the case in teleost model system. However the expression of MC1R in human steroid producing tissues has not been extensively studied, which should be included in future studies.–Moving forward it will be interesting to analyse data from the complete data set. Furthermore if granted permission to implement a stress test and base the selection regime of HCP and LCP on stress responses (as well as CAR), it will be possible to further validate

whether CAR is indicative of HPA-axis reactivity and assessing whether different genotypes are correlated with CAR dynamics and/or stress/HPA-Axis reactivity.

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7 Appendix

A.1 Demographic details of study population

	Complete study population		Study participants with extended CAR measurement
	Start up (n=126)	**2-week** (n=124)	**Start up** (n=34)
Age Mean (SD), years	44 (12,4)	43,1 (11,9)	45,3 (16,4)
sex, n, F:M	92:33 *	85:39	24:10
Educational level, ISCED (SD)	5,9 (1,4)	5,8 (1,4)	5,32 (1,8)
Comorbid disorders, n	64	60	19

Table 3: demographic details of complete study population and sub-set with 5 morning samples measuring extended CAR. *Some participants were missing demographic data

	Start up complete population-sample			**Start up subset with extended CAR sampling**		
	ll **(n=41)**	***ls*** **(n=69)**	***ss*** **(n=25)**	***ll*** **(n=10)**	***ls*** **(n=15)**	***ss*** **(n=8)**
Age Mean (SD), years	42,5 (12,6)	45 (12,1)	41,6 (12,2)	34,2 (11,1)	50,07 (16)	44,63 (17,5)
sex, n, F:M	28:13	43:25 *	21:5	8:2	10:5	6:2
Educational level, ISCED (SD)	5,9 (1,4)	6 (1,4)	5,6 (1,6)	4,8 (2)	6,07 (1,4)	5,20 (1,7)
Comorbid disorders, n	20	32	12	6	9,00	4,00

Table 4: Demographic details from carriers of each 5-HTTLPR genotype. *Some participants were missing demographic data.

	HCP (n=12)	**LCP** (n=13)
Age Mean (SD), years	46,5 (17,4)	41,8 (14,9)
sex, n, F:M	7:3 *	08:05
Educational level, ISCED (SD)	5,7 (1,3)	5,85 (1,2)
Comorbid disorders, n	5	7

Table 5: Demographis details of HCP vs. LCP groups. *two participants were missing demographic data.

A.2

Comparison	Difference	P value
E1 vs. M1a	-142.95	*** P<0.001
E1 vs. M1b	-180.91	*** P<0.001
E1 vs. M1c	-169.54	*** P<0.001
E1 vs. M1d	-163.10	*** P<0.001
E1 vs. M1e	-149.66	*** P<0.001
M1a vs.M1b	-37.959	ns P>0.05
M1a vs.M1c	-26.592	ns P>0.05
M1a vs.M1d	-20.155	ns P>0.05
M1a vs.M1e	-6.713	ns P>0.05
M1b vs. M1c	11.367	ns P>0.05
M1b vs. M1d	17.805	ns P>0.05
M1b vs. M1e	31.247	ns P>0.05
M1c vs. M1d	6.438	ns P>0.05
M1c vs. M1e	19.880	ns P>0.05
M1d vs. M1e	13.442	ns P>0.05

Table 5: Kruskal-Wallis Test (Nonparametric ANOVA). Dunn's Multiple Comparisons Test

A.3 Solutions and buffers for RIA

Antibody Coating Buffer

$NaHCO_3$	*50mM*	*(4.2g/Liter)*
Na_2CO_3	*50mM*	*(5.4g/Liter)*
NaN_3	*0.02%*	*(0.2g/Liter)*

pH 9.6

Wash Buffer

Tris	*100mM*	*(12g/Liter)*
NaCl	*0.9%*	*(9g/Liter)*
NaN_3	*0.02%*	*(0.2g/Liter)*

pH 7.4

Assay Buffer

Tris	*100mM*	*(12g/Liter)*
NaCl	*0.9%*	*(9g/Liter)*
ANS	*0.1%*	*(1g/Liter)*
NaN_3	*0.02%*	*(0.2g/Liter)*

pH 7.4

Cortisol Antibody

(Abcam: ab1949; Cortisol Antibody[xm210] monoclonal and IgG purified)
Diluted 1:2000 in Coating Buffer. Prepared freshly and used immediately.

Blocking Buffer (0.25%)

250µl Normal Calf Serum (NCS) in 100ml Wash Buffer.

Cortisol Standard

1µg/10µl Cortisol in EtOH *(Sigma: H4001-5G; Hydrocortisone ≥98% HPLC)*
Add 990µl Assay Buffer (gives 1µg/1000µl = 10,000pg/10µl) and vortex.
Take out 204.8µl and add 795.2µl Assay Buffer to obtain 2048pg/10µl Cortisol Standard
Dilute serially (1:1) to obtain standards 2048-1024-512-256-128-64-32-16-8-4 pg.

Cortisol tracer *(PerkinElmer: #NET396250UC - Hydrocortisone (Cortisol,[1,2,6,7-^{3}H(N)]-),[1,2,6,7-^{3}H(N)]- 250*µCi(9.25MBq)
^{3}H-Cortisol is stored at the radioactive laboratory (P. Cruijsen) in the freezer.
- Per plate: add 1µl ^{3}H-Cortisol tracer to 10ml Assay Buffer

A.4 Solutions for PCR

For MC1R PCR reaction manufacture of buffers and Taq polymerase was Invitrogen. See also table 1 for details of reaction mix and PCR cycling instructions.

A.5 Solutions and buffers for 1,5 % Agarose

50X TAE buffer 1L

Tris(hydroxymethyl)aminomethane (Tris)-base	242 g
Acetic acid	57.1 ml
0.5M sodium EDTA	100 ml
Add H_2O to make total volume 1 L	<1L

To make 1x TAE from 50X TAE stock, dilute 20ml of stock into 980 ml of DI water pH 8.3

1,5 % Agarose gel

TAE buffer	100*ml*	
Agarose	1,5 *g*	*(1,5g/dL)*
Gel Red	*0.008%*	*(8 µL/dL)*