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Exploring Links between Melatonin, Inflammation and Depression

ISAK SUNDBERG



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Abstract

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Major depressive disorder (MDD) is one of the leading global causes of disease burden. Worse yet, about one third of the patients with MDD do not experience a remission with current treatments. The symptoms of MDD likely represent a variety of underlying pathologic processes and more knowledge about these processes is needed to optimize treatment for MDD. The focus of this thesis was to study the relationship between inflammation, melatonin and symptoms of depression.

In papers I-III a population of young adults seeking psychiatric care was examined for depressive symptoms, melatonin levels in saliva, gastrointestinal (GI) symptoms and inflammatory markers in blood. In paper IV a cohort of patients with hepatitis C receiving treatment with new direct-acting agents (DAAs) were prospectively followed during treatment for depressive symptoms and sleep.

All patients were diagnosed by means of structured or semi-structured interviews and depressive symptoms were assessed with the self-rating version of the Montgomery Åsberg Depression Rating Scale. Sleep quality was measured by the Pittsburgh Sleep Quality Index, and GI symptoms were assessed with the Gastrointestinal Symptom Rating Scale-IBS. Melatonin in saliva was measured using enzyme-linked immunosorbent assay, and inflammatory markers in blood were analysed by proximity extension assay.

In young adults seeking psychiatric care melatonin levels at bedtime were inversely correlated with depressive symptoms. In those patients with a current depressive episode low melatonin values at bedtime were a negative prognostic factor for response after 6 months (paper I). Postprandial melatonin levels were positively associated with GI symptoms of bloating and pain (paper II). Postprandial melatonin levels were also associated with the inflammatory markers vascular endothelial growth factor A (VEGF-A), monocyte chemoattractant protein-1 (MCP-1) and monocyte inflammatory protein-1 α (MIP-1 α). Evening levels of melatonin did not correlate with the inflammatory markers. VEGF-A and MCP-1 as well as postprandial levels of melatonin correlated with a diagnosis of anxiety disorder, whereas MIP-1 α correlated with MDD (paper III). Patients with hepatitis C underwent treatment with DAAs without experiencing pronounced psychiatric side effects in terms of depressive symptoms or sleep disturbances (paper IV).

In summary, the findings confirm a relationship between bedtime melatonin levels and depressive symptoms. The findings also show a connection between daytime melatonin and GI-symptoms. In addition, the findings indicate an association between inflammation and daytime melatonin. Together these results demonstrate links between melatonin, inflammation and depression. Lastly, interferon-free treatment against hepatitis C did not induce depressive symptoms.

Keywords: melatonin, inflammation, depression, biomarkers, cytokines, anxiety, hepatitis C

Isak Sundberg, Department of Neuroscience, Psychiatry, University Hospital, Akademiska sjukhuset, Uppsala University, SE-751 85 Uppsala, Sweden.

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Know then thyself, presume not God to scan;
The proper study of mankind is man.
Plac'd on this isthmus of a middle state,
A being darkly wise, and rudely great:
With too much knowledge for the sceptic side,
With too much weakness for the stoic's pride,
He hangs between; in doubt to act, or rest;
In doubt to deem himself a god, or beast;
In doubt his mind or body to prefer;
Born but to die, and reas'ning but to err;
Alike in ignorance, his reason such,
Whether he thinks too little, or too much:
Chaos of thought and passion, all confus'd;
Still by himself abus'd, or disabus'd;
Created half to rise, and half to fall;
Great lord of all things, yet a prey to all;
Sole judge of truth, in endless error hurl'd:
The glory, jest, and riddle of the world!

Essay on Man, Epistle II
Alexander Pope, 1732

To my family and to my patients

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I. Sundberg I, Ramklint M, Stridsberg M, Papadopoulos FC, Ekselius L, Cunningham JL. (2016) Salivary Melatonin in Relation to Depressive Symptom Severity in Young Adults. *PLoS One*. 11(4):e0152814. doi: 10.1371/journal.pone.0152814.
- II. Söderquist F, Sundberg I, Ramklint M, Widerström R, Hellström PM, Cunningham JL. (2018) The Relationship Between Daytime Salivary Melatonin and Gastrointestinal Symptoms in Young Adults Seeking Psychiatric Care. *Psychosom Med*. Oct 6. doi: 10.1097/PSY.0000000000000644.
- III. Sundberg I, Rasmusson A, Ramklint M, Ekselius L, Cunningham JL. Daytime Melatonin Levels in Saliva are Associated with Inflammatory Markers and Anxiety Disorders in Young Adults with Psychiatric Disease. *Manuscript*.
- IV. Sundberg I, Lannergård A, Ramklint M, Cunningham JL. (2018) Direct-acting antiviral treatment in real world patients with hepatitis C not associated with psychiatric side effects: a prospective observational study. *BMC Psychiatry*. May 29;18(1):157. doi: 10.1186/s12888-018-1735-6.

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Abbreviations

AANAT	Serotonin N-acetyltransferase
AUDIT	Alcohol Use Disorders Identification Test
BBB	Blood–brain barrier
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
COX-2	Cyclo-oxygenase-2
CRP	C-reactive protein
CTT	Colonic transit time
DAA	Direct-acting antiviral agents
DSM	Statistical Manual of Mental Disorders
DUDIT	Drug Use Disorders Identification Test
EC	Enterochromaffin
ECT	Electroconvulsive treatment
ELISA	Enzyme-linked immunosorbent assay
GAD	Generalized anxiety disorder
GI	Gastrointestinal
GSRS-IBS	Gastrointestinal Symptom Rating Scale-IBS
HCV	Hepatitis C virus
IBS	Irritable bowel syndrome
IFN	Interferon
IL	Interleukin
KYNA	Kynurenic acid
LPS	Lipopolysaccharides
LRT	Likelihood ratio test
MADRS	Montgomery-Åsberg Depression Rating Scale
MCP-1	Monocyte chemoattractant protein-1
MDD	Major depressive disorder
M.I.N.I.	M.I.N.I. International Neuropsychiatric Interview
MIP-1 α	Monocyte inflammatory protein-1alpha
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIMH	National Institute of Mental Health
NSAID	Non-steroidal anti-inflammatory drug
OCD	Obsessive-compulsive disorder
OR	Odds ratio
PDGF	Platelet-derived growth factor

PEA	Proximity extension assay
PSQI	Pittsburgh Sleep Quality Index
PTSD	Post-traumatic stress disorder
QUIN	Quinolinic acid
RDoC	Research Domain Criteria
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rTMS	Repetitive transcranial magnetic stimulation
SAD	Social anxiety disorder
SCID-1	Structural Clinical Interview for DSM IV-axis I
SCN	Suprachiasmatic nucleus
SSRI	Selective serotonin reuptake inhibitor
SVR	Sustained viral response
TNF- α	Tumor necrosis factor-alpha
TLR-4	Toll like receptor -4
TPH	Tryptophan hydroxylase
TRD	Treatment resistant depression
VEGF-A	Vascular endothelial growth factor-A
5-HT	Serotonin

Introduction

A clinical case of interferon-induced depression

A couple of years ago, a patient (hereafter referred to as Patient X) is about to receive treatment for hepatitis C. He is one of about 185 million patients in the world with this viral infection. Patient X presents with clinical symptoms of malaise and slight difficulties concentrating. In addition, there is the psychological burden of having a chronic disease and living with the elevated risk of developing other severe liver diseases such as end-stage liver disease and hepatocellular cancer.

He could have acquired the virus during a blood transfusion while in surgery, but more likely caught it via intravenous drug abuse. He had already been administered the most common treatment against hepatitis C, namely pegylated interferon alpha (IFN- α). At that time, he was one of three patients who developed depression during treatment and therefore he had to terminate therapy because of severe side effects.

This time, after about a week of interferon treatment, his sleep begins to show deterioration: he feels sick, exhibits a lack of motivation and nothing seems interesting or eventful anymore. Patient X now feels constantly depressed and anxious, being unable to function properly at work or in his social life. These symptoms continue for a couple of weeks and the patient starts to experience suicidal thoughts.

We followed this man from baseline before treatment with interferon. We performed a thorough psychiatric evaluation with structured diagnostics. When we looked at how depressive symptoms, melatonin and inflammatory cytokines developed during the treatment period, it appeared like that shown in the figures below.

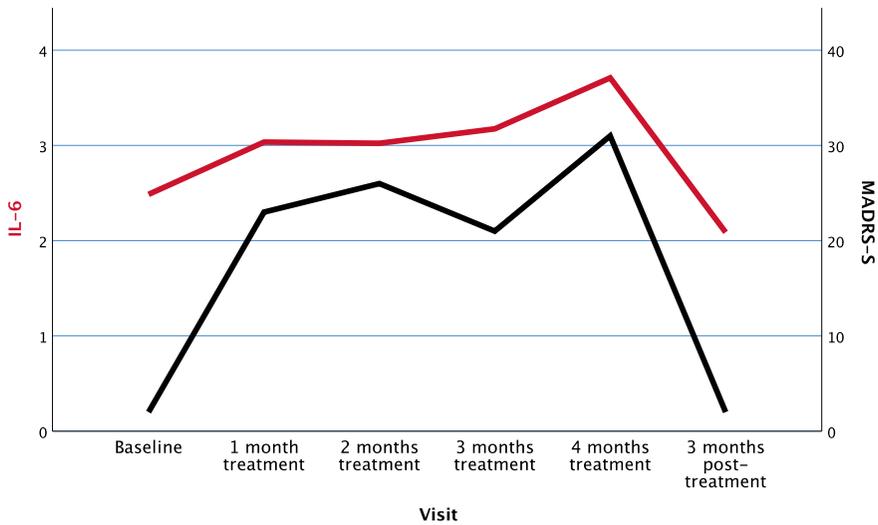


Figure 1. The pro-inflammatory cytokine interleukin-6 (IL-6) in blood in relation to depressive symptoms (MADRS-S) during treatment with IFN- α , from baseline to three months post-treatment.

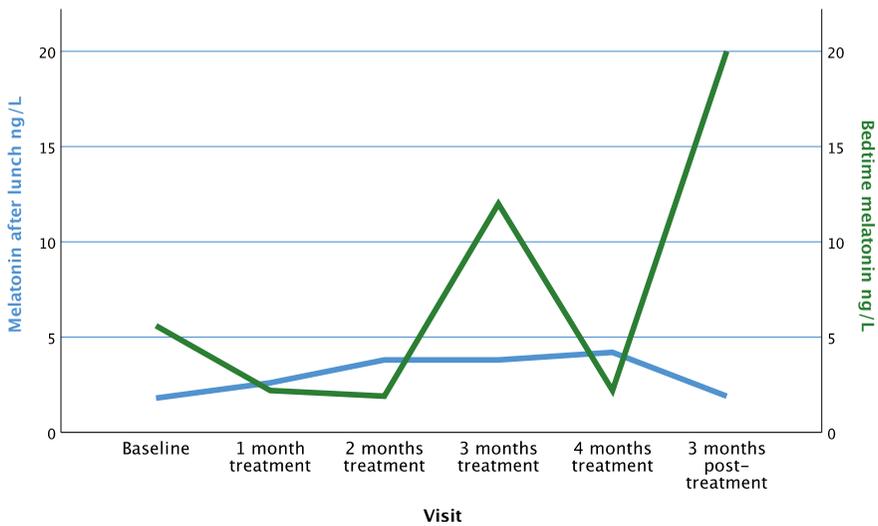


Figure 2. Levels of melatonin in saliva after lunch and at bedtime during treatment with IFN- α , to three months post-treatment.

In this patient, with interferon-induced depression, we were able to study the longitudinal connection between inflammatory markers, melatonin and depressive symptoms. The inflammatory cytokine IL-6 rises during treatment with interferon, as does depressive symptoms. Melatonin after lunch goes up during treatment, while bedtime melatonin falls, suggesting a shift in the

production of melatonin. It illustrates how interferon treatment has been used as a model for cytokine-induced depression and brings us to the motivation and scope of this thesis.

Major depressive disorder (MDD) is predicted to become the leading global cause of disease burden in the next decades. The syndrome called MDD may actually reflect several underlying biological processes. With today's treatment arsenal, about one third of patients with depression do not attain remission (1). Disruption in endocrine and inflammatory response systems are pathophysiological mechanisms implicated in the etiology of MDD. This thesis explores the association and interaction between these systems and MDD. Specifically, we examine the role of the hormone melatonin in relation to depressive symptoms, metabolic regulation, GI symptoms and biomarkers related to the immune system.

The studies in this doctoral thesis are performed in young adults with psychiatric disease and in patients with hepatitis C during treatment with new direct-acting antiviral drugs, where the psychiatric side effects of these drugs are examined.

Background

Depression

Major depressive disorder (MDD) is different from usual mood fluctuations and short-lived emotional responses to challenges in everyday life. MDD may become a serious health condition if it is long-lasting and of severe intensity in that it can cause considerable suffering and poor functionality (e.g., at work, at school and in the family). By 2030, MDD is expected to become the leading cause of disease burden worldwide (2). Depression is a common underlying cause of suicidality; in fact, close to 800 000 people die worldwide because of suicide every year and suicide is the second leading cause of death in adolescents and young adults (3). MDD influences the outcome of many other somatic diseases and patients with psychiatric disease have a higher risk of metabolic disease and early death compared with the general population (4). In people with a previous diagnosis of unipolar depression the overall mortality rate ratio was 2.09 compared with the general population, which translates into a shorter life expectancy of 14 years in men and 10 years in women (5).

Until recently, pharmacological treatment for depression has largely centered on medications altering uptake of monoamines. Regrettably, about one third of patients treated for depression do not attain remission, increasing the risk for chronic depression and eventual suicide. Electroconvulsive therapy (ECT) is another treatment option and has often proven effective in severe depression. Repetitive transcranial magnetic stimulation (rTMS) has come into routine practice in some countries and Ketamine infusion is one example of a promising addition to the clinical armamentarium against MDD.

The Diagnostic and Statistical Manual of Mental Disorders (DSM) category of depression most likely represents numerous underlying heterogeneous pathophysiological conditions (6). Relating to this, the Research Domain Criteria (RDoC), initiated by the National Institute of Mental Health (NIMH), is a research framework for new ways of studying mental disorders based on dimensions of observable behavior and neurobiological measures (7). The goal is to integrate many levels of information (from genomics to self-report) to better understand basic dimensions of functioning that cut across traditional disorder categories in psychiatry. Such an approach can be

used as the basis for grouping patients in clinical studies and may to a larger extent reflect underlying pathological mechanisms.

Given the enormous clinical burden of depression and the shortcomings of current treatments and diagnostic approaches, there is a continuous need to find categories of MDD based on these biological underpinnings to more adequately study depression. It is also important to find subgroups, with implications for which patients to treat best with existing therapies and to find new therapeutic targets in a more efficient and rational way (8).

In this quest to understand the pathophysiology of the different variants of depressive disorders, to personalize treatments and to ultimately discover new treatments against depression, several converging areas of research are of interest. These areas include inflammation (brain and periphery), the connection between the gut and the brain and the relationship between melatonin, the immune system and depression.

Cytokines

Cytokines are soluble messengers that can communicate between the innate and the adaptive immune system. Chemokines are a subset of cytokines with chemotactic ability, i.e. they can recruit special cells to a site. Cytokines bind to specific receptors on the membranes of target cells, triggering signal transduction pathways that ultimately alter enzyme activity and gene expression. Monocytes can differentiate into specific tissue macrophages. Activated, inflammatory macrophages have increased phagocytic activity and recreation of inflammatory and cytotoxic mediators. Macrophages in the central nervous system (CNS) are called microglia.

Melatonin

Melatonin was originally discovered 50 years ago by the American dermatologist Aaron Lerner and his co-workers as an amphibian skin-lighting factor present in extracts of bovine pineal glands. Lerner named the molecule melatonin because it induces contraction of stellate amphibian melanophores (9). Melatonin is present in a wide spectrum of organisms from bacteria to man. The fact that melatonin is an evolutionarily highly conserved molecule implies its important physiological role (10).

Melatonin is derived from the essential amino acid tryptophan via serotonin (see Figure 1). The rate of melatonin formation depends on the activity of two enzymes: serotonin N-acetyltransferase (AANAT) and, to a lesser extent, tryptophan hydroxylase (TPH), which controls the availability of serotonin (10).

TPH is a mitochondrial enzyme that transforms tryptophan to 5-hydroxytryptophan. It exists in two isoforms: TPH1 and TPH2. TPH1 is found in the pineal gland and the gut, whereas TPH2 is expressed primarily in the brain (11). In the pineal gland and retina the expression of TPH fluctuates in a clock-driven circadian rhythm, with peak values during the night period. The nocturnal increase in the enzyme activity requires *de novo* protein synthesis (10).

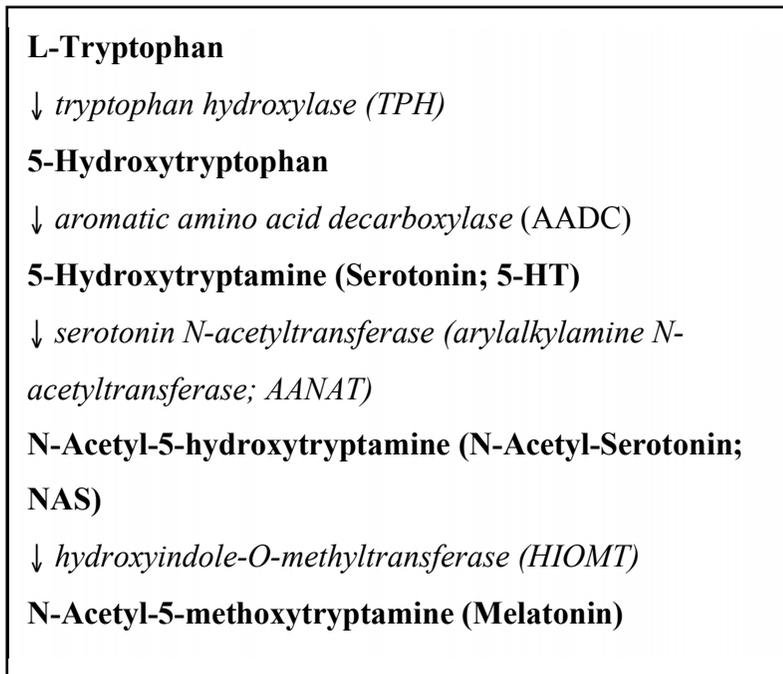


Figure 3: Melatonin synthesis pathway

Because of its role as a key regulatory enzyme in the melatonin bio-synthetic pathway, AANAT has been named “the melatonin rhythm enzyme” (12). Changes in melatonin content reflect oscillations in AANAT activity. A master circadian clock located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus controls pineal AANAT activity. The dynamic changes in AANAT activity in this context are regulated by control systems that consist of two basic elements: an autonomous circadian clock and turn-off mechanisms (12). Information about light is transmitted from the retina to the hypothalamus: during the day, in the presence of light, the output from the retino-hypothalamic tract inhibits melatonin synthesis by turning off the production of AANAT (13).

Because melatonin is not stored in the pineal gland, plasma levels of melatonin closely reflect pineal activity (at least during the night) (10). Melato-

nin is amphiphilic, i.e. it is soluble in both water and lipid. Therefore, circulating melatonin can reach all body tissues, including the brain, and is able to cross the blood-brain barrier (BBB) to modulate brain activity (14).

The liver deactivates more than 90% of circulating melatonin and the half-life of melatonin after exogenous administration in humans is between 10 and 60 minutes (min) (10). The average maximum levels attained in the plasma of adults are from 60 to 70 pg/ml, but melatonin levels decline markedly with age (15). The concentrations of melatonin in saliva have been shown to significantly correlate with blood levels of melatonin (16).

The peak concentrations of melatonin in plasma normally occur between 02.00 and 04.00 hours (h); the onset of secretion is usually around 21.00 to 22.00 h and the offset at 07.00 to 09.00 h in adults in temperate zones (10).

The normal human melatonin rhythm is very stable over time in normal individuals, almost like a hormonal fingerprint (17). Still, there are large individual differences in the amplitude of the melatonin rhythm (17).

Melatonin acts via G-protein-coupled membrane receptors (e.g., MT1, MT2, MT3) that modulate several intracellular messengers, such as cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP) and $[Ca^{2+}]$. These receptors are expressed in the brain (SCN, hippocampus, cerebellum), retina, immune system (lymphocytes), brown adipose tissue, salivary glands, cardiovascular system, breasts, kidneys, pancreas, GI tract, gallbladder, ovary, uterus, prostate and the skin (18). Melatonin can also bind to nuclear receptors (19) and melatonin or its metabolites may indirectly modulate nuclear receptors (20).

Mitochondria and melatonin

The precursor of mitochondria is believed to be the purple nonsulfur bacterium (particularly, *Rhodospirillum rubrum*). This bacterial species was in symbiosis with host proto-eukaryotes and gradually transformed into cellular organelles (i.e. mitochondria), thereby giving rise to eukaryotic cells. Purple nonsulfur bacteria synthesize melatonin. Mitochondria were likely the original sites of melatonin synthesis in the early stage of endosymbiotic organisms and the capacity to produce melatonin was carried into host eukaryotes by the bacteria mentioned above. The ability to produce melatonin in other cellular compartments may have derived from mitochondria (21).

Mitochondria are known as the powerhouse of the cell, generating reactive oxygen species (ROS) and free radicals while producing energy. Because of this, these organelles require strong protection from free radicals and associated oxidative stress. Melatonin is a potent free radical scavenger and antioxidant. The high levels of melatonin produced by mitochondria are used to protect this important cellular organelle against oxidative stress and preserve its physiological functions (21).

Pineal and extra-pineal melatonin production

Functionally, melatonin production can be divided into pineal and peripheral or extra-pineal production. The function of pineal melatonin is that of conveying photoperiodic information, which maintains circadian and other biological rhythms. The major function of extra-pineal melatonin seems to be that of counteracting oxidative stress and inflammation.

Extra-pineal melatonin

Although the density of melatonin receptors is higher in pinealocytes than in peripheral tissues, the total amount of melatonin as well as local concentrations in the periphery can exceed pineal melatonin by several magnitudes (22). Most of the peripheral melatonin is thought to act foremost locally, with lesser amounts reaching the blood stream. Except for in the retina, peripheral melatonin appears to be produced in a non-circadian manner (23).

The GI tract is the most abundant extra-pineal source of circulating melatonin with mucosal concentrations exceeding blood plasma levels by 100-400 times (24). Melatonin is produced in the GI tract during the daytime, especially after meals with high content of tryptophan (25). The pancreas is another source of melatonin, which may also contribute to the total levels measured in blood or saliva (18). During the daytime, circulating levels of melatonin are believed to be of peripheral origin (22). The innate immune system, and actually all mitochondria containing cells, are capable of producing melatonin (26, 27).

Melatonin and depression

Wetterberg and colleagues were among the first in the 1980s to demonstrate lower peripheral nighttime and/or 24-h melatonin levels in depressed patients compared with healthy controls, which has been replicated by others (28-34). These studies have generally been conducted on inpatients with severe depression. Other studies, in outpatient settings (35, 36) or with mixed patient groups (37), have not found significant differences in melatonin levels between patients with depression and controls. There are also reports of higher melatonin concentrations in depressed individuals as compared with healthy controls (38). Negative correlations between depression severity and 24-h amplitude of plasma melatonin have been reported (32). In addition, a positive correlation was found between depression severity and degree of circadian misalignment (39). A negative correlation between evening melatonin levels and the degree of depressed mood and reality disturbance has also been reported (40). Other publications have noted no significant relationship between levels of melatonin and indices of depression se-

verity (35, 38, 40-42). It is relevant to note that most studies have been conducted with small sample sizes with a limited range of depression severity. Furthermore, potential confounding factors such as age (43, 44), sex (45), use of oral contraceptives (46), antidepressant medication (47), BMI (48, 49), beta blockers (50) and season (10) are often not addressed in these studies.

Melatonin and inflammation

During the course of inflammation, lipopolysaccharides (LPSs) and peripheral cytokines, such as tumor necrosis factor (TNF)- α , shut down pineal melatonin production, contributing to the decreased pineal circadian melatonin synthesis across many medical conditions (51, 52). This can be mediated by TNF- α effects directly in pinealocytes or indirectly via toll-like receptor (TLR)-4 activation in microglia around the pineal gland, which drives local TNF- α production and release (52).

Melatonin may be regulated differently in peripheral tissues where it has autocrine functions, switching macrophages to a less inflammatory, phagocytic M2-like phenotype (26). *In vitro*, the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathway is the downstream mechanism that links the stimulation of macrophages by danger signals directly to the control of AANAT. In turn, macrophage-synthesized melatonin enhances phagocytosis through autocrine action (53).

In the context of the immune-pineal axis the NF- κ B pathway thus plays a dual role depending on the cellular environment. Danger signals lead to an inhibition of melatonin synthesis in the pineal gland, whereas they induce the synthesis of melatonin by macrophages. These apparently opposite effects are important for the initiation and resolution of the innate immune response, largely because a reduction in the nocturnal melatonin surge will allow leukocyte migration to sites of injury, whereas the production of melatonin by immune-competent cells (independent of the circadian rhythm) is important for pathogen clearance (53).

Additionally, melatonin has been shown to have a wide spectrum of regulatory metabolic functions (54). Besides direct scavenging of ROS/reactive nitrogen species (RNS), melatonin stimulates antioxidant enzymes, suppresses pro-oxidant enzymes and improves mitochondrial function, all of which reduces radical formation (20, 55).

Melatonin can also influence transcription factors involved in insulin secretion in the pancreas (54, 56, 57). There also seems to be a link between melatonin and glucose homeostasis (58, 59). Recently, Söderquist and co-workers described mRNA and protein expression of melatonin receptors (MT2 and MT1) in the human pancreas (18). Genetic variants of MT2 are reported to increase the risk of developing type 2 diabetes. More receptors

on the cell surface increase the sensitivity for melatonin, an event that reduces the capacity to release insulin (60, 61).

While melatonin is most known for its anti-inflammatory properties, depending on various conditions, including cell types, level and duration of inflammation, it can also have pro-inflammatory effects (20). Melatonin appears to have a pro-inflammatory role foremost at an early phase in the inflammatory response; it has an antagonist role at later phases, given that it can activate pro-inflammatory mediators such as interleukin-1 (IL-1) and TNF- α at an early stage of inflammation but in later stages down regulate the same mediators (19). Pro-inflammatory actions of melatonin enhance the resistance against pathogens but may be detrimental in some autoimmune diseases (e.g., rheumatoid arthritis) (62).

In summary, the interaction between melatonin and the immune system is complex, extending well beyond anti-oxidative effects (20). The bidirectional relationship between melatonin and the immune system has been termed the immune-pineal axis (63, 64).

Few studies have investigated potential associations between endogenous daytime levels of melatonin and cytokine levels in clinical settings. When patients with newly diagnosed multiple sclerosis were compared with healthy controls, morning serum levels of melatonin were lower and TNF- α higher, but not significantly correlated with each other (65). Patients with chronic renal failure had lower melatonin levels than those of controls and melatonin levels were negatively correlated with TNF- α (66). In contrast, patients with polymyalgia rheumatica had higher levels of melatonin compared with controls, with the relative difference being most pronounced during the day (62). These studies indicate an association between melatonin and serum inflammatory markers but the studies differ in sampling times, which may explain some of the discrepancies.

Inflammation and depression

Inflammation is a biological host defense mechanism characterized by increased blood flow and recruitment of innate immune cells to the site of injury. The link between increased inflammation and depression was detected in the early 1990s, leading to the formulation of the macrophage hypothesis of depression or the cytokine hypothesis of depression (67). This model proposes that external and internal stressors trigger depressive behavior by elevating the production of pro-inflammatory cytokines (e.g., IL-1 and IL-6), as well as activating cell-mediated immunity. More recently, an abundance of observational, experimental and clinical evidence has emerged to suggest that the activation of innate immune mechanisms, may contribute to the initiation and progression of such psychiatric diseases as depression (68, 69). Particularly noteworthy is that psychological stress may also activate key

inflammatory pathways (in peripheral blood mononuclear cells), including activation of the transcription factor NF- κ B, leading to increased levels of circulating pro-inflammatory cytokines (70). The greater this inflammatory response, the greater the risk of depression in the coming months (71). Moreover, the relationship between inflammation and depression is influenced by sex, being generally more pronounced in females (72).

Links between inflammation and depression

There are several links between inflammation and depression. As a group, patients with MDD have higher levels of certain pro-inflammatory cytokines compared with controls (73). They also show higher levels of markers of oxidative stress (74). The most consistent finding in cytokines and depression is elevated levels of IL-1, IL-6 and TNF- α in patients with depression compared with controls (75). However, IL-10, the soluble IL-2 receptor, C-C chemokine ligand 2, IL-13, IL-18, IL-12, the IL-1 receptor antagonist and the soluble TNF receptor 2 have also been found to be elevated in patients with MDD (76).

In one study higher IL-6 levels in childhood were associated with subsequent risks of depression in a dose-dependent manner (77). Prospective studies have shown that there may be a normalization of overactive inflammatory processes following antidepressant treatment, with elevated pro-inflammatory cytokines normalized after recovery from depression (78, 79). The direction of the relationship insinuates that inflammation induces depression, although there are reports of the opposite direction: depression preceding elevated inflammatory markers (80).

There is substantial heterogeneity, however, in studies examining the relationship between inflammatory cytokines and depression and several studies have produced mixed or negative results (81, 82).

Mechanisms

The mechanisms linking inflammation and cytokines to depression are not fully understood. However, a few possible mechanisms merit mention here.

IL-1B and TNF- α have been shown to increase the expression and function of the reuptake pumps for serotonin, leading to decreased synaptic availability of serotonin and depressive-like behavior in laboratory animals (83).

Inflammatory cytokines (e.g., IL-17) generate ROS and increase permeability of the BBB that result in the infiltration of immune cells. They also increase inflammation by stimulating microglia production of yet more pro-inflammatory cytokines in a potentially vicious cycle (84).

The kynurenine pathway is an important possible mechanism for how inflammation may cause depressive symptoms. Tryptophan is metabolized by

several biochemical pathways; however, more than 90% of tryptophan is metabolized through the kynurenine pathway and changes in this pathway are recognized as a mechanism by which depressive symptomatology can develop (85). Kynurenic acid (KYNA) is an NMDA receptor antagonist that has anti-oxidative and neuroprotective properties, whereas quinolinic acid (QUIN) is an NMDA receptor agonist and may stimulate lipid peroxidation and thereby is neurotoxic. Enhanced kynurenine influx from the periphery apparently increases the ratio between the neurotoxic metabolite QUIN and the neuroprotective KYNA. This pathway is influenced by pro-inflammatory cytokines. Prospective studies of patients with Hepatitis C who received IFN treatment showed increased neurotoxic challenge (kynurenine to KYNA quotient, higher levels of QUIN) that was related to depressive symptoms and mediated by greater activity of indoleamine 2,3-dioxygenase (86-89). Theoretically, less tryptophan available for production of serotonin could also be one mechanism by which depressive symptoms are increased. However, some evidence speaks against this mechanism (90). Taken together, enhanced levels of QUIN, increasing neurotoxicity may result in depressive symptoms.

Inflammation, depression and treatment

There are reports of differential cytokine and chemokine production in groups with different genesis or prognosis of depression. It has been suggested that dysthymia has a different cytokine milieu compared with MDD (91), that cytokine profiles differ between patients who are and who are not early responders to the antidepressant duloxetine (92) and that the differential immunomodulatory effects of different drugs could be used to tailor treatment to specific individuals according to their immune endophenotypes (93)(94). Higher levels of platelet-derived growth factor (PDGF) predicted better outcomes with a combination of bupropion-selective serotonin reuptake inhibitors (SSRIs) than SSRI alone (95). Adjunctive treatment with non-steroidal anti-inflammatory drugs (NSAIDs) to anti-depressant treatment has been tested with some positive results (96).

There is evidence of antidepressant activity of anti-cytokine treatment (97, 98). An often-cited proof-of-concept study is one in which patients with treatment-resistant depression (TRD) received treatment with an inhibitor of TNF- α (infliximab). Post-hoc analyses showed that those patients with CRP above 5 mg/L benefitted from the treatment (99).

Taken together, these findings underscore a potentially causal role for cytokines in depression and that cytokine modulators may be novel drugs for depression in patients with chronic inflammation (97).

Melatonergic drugs

Agomelatine is an antidepressant drug acting as MT1 and MT2 receptor agonist and as 5HT2C receptor antagonist. Reduction of both TNF- α (100) and CRP has been connected with the anti-depressant effect of agomelatine (101).

Inflammation and anxiety

Depression and anxiety disorders share some signs and symptoms but each disorder has its own causes as well as different emotional and behavioral symptoms. The most common forms of anxiety disorder are briefly described below. In *social anxiety disorder* (SAD) a person has excessive concern about negative evaluation leading to marked anxiety in, or avoidance of, social situations. *Obsessive-compulsive disorder* (OCD) is a disorder in which a person has uncontrollable, reoccurring thoughts (obsessions, such as fear of germs or contamination) and behaviors (compulsions, such as excessive cleaning, hand washing, or both) that he or she feels the urge to repeat over and over. *Post-traumatic stress disorder* (PTSD) entails persistent symptoms of hyperarousal, intrusive memories and avoidance of stimuli associated with a traumatic event. (Now the diagnosis of PTSD has moved to the new DSM-V category Trauma- and stressor-related disorders, but when patients were recruited for this study, DSM-IV was in use.)

Generalized anxiety disorder (GAD) is characterized by persistent and excessive anxiety and worry (apprehensive expectation) that occur more often than not for at least 6 months about everyday life events (e.g., work, family, money, school performance). Individuals with GAD find it difficult to control their anxiety and worry. These concerns are often accompanied by physical symptoms.

Anxiety disorders have a high contribution to the global burden of disease, estimated to be the sixth leading cause of disability worldwide (102). The relationship between inflammation and anxiety is less well explored than that between inflammation and depression. However, in a recent review and meta-analysis the association between anxiety, traumatic stress and OCD and chronic inflammation was explored (103). Higher levels of IL-1 β , IL-6 and TNF- α were found in patients compared with controls (i.e. including the same markers also most strongly connecting inflammation with depression). One recent study found that anxiety preceded a rise in inflammatory markers (e.g., IL-6), which preceded depressive symptoms in a cohort of adolescents (104).

The gut-brain axis

High rates of comorbidity between GI and psychiatric illnesses suggest a potential contribution of bidirectional communication between the gut and CNS (105, 106). For example, mood disorders affect more than half of all patients with irritable bowel syndrome (IBS) (107) and antidepressants are one of the most common medications for IBS (105). The bidirectional communication network that links gut functions to cognitive and emotional centers of the brain has been called the gut–brain axis. This axis mediates the effects of both genetic and environmental factors on brain development and function and has been implicated in the etiology of a number of psychiatric disorders (108).

Gut permeability as a potential source of inflammation

Gut permeability increases through loosening of the tight junctions that closely link the cells lining the gut. A number of factors have been shown to increase gut permeability, including dietary fats, stress and alcohol. Inversely, a number of factors can decrease permeability or help to maintain gut tight junction integration, including dietary whole grains and melatonin (109). With increased gut permeability, LPS from the walls of Gram-negative gut bacteria can translocate through the intestinal wall, thereby activating the innate immune system and ultimately drive increased depressive symptoms through various mechanisms (110). These mechanisms include the activation of NF- κ B, a transcription factor that drives many inflammatory genes and processes, including TNF- α , IL-1 β and cyclooxygenase-2 (COX-2). Markers of gut permeability have also been associated with increased suicidal behavior (111).

Production of melatonin in the gut

The enterochromaffin (EC) cells are neuroendocrine cells located throughout the GI tract that produce and secrete serotonin and melatonin, which are two enzymatic steps away from each other. EC cells are thus a major source of melatonin in the GI tract (22, 112).

Melatonin affects GI motility via membrane receptors that include melatonin (MT1 and MT2) and serotonin (5-HT) receptors (113). Melatonin induces contraction of cultured gastric smooth muscle cells, likely via MT1 receptor signaling (114). Binding of melatonin to 5-HT₄ receptors can cause smooth muscle relaxation, whereas stimulation of 5-HT₃ receptors may result in smooth muscle contraction (113). Melatonin may also influence gut smooth muscle via the inhibition of nicotinic receptor channels regulating smooth muscle contraction (113). Furthermore, melatonin can inhibit the activity of the serotonin transporter (115).

The reported effects of melatonin on GI motility appear to be dose dependent, as administration of pharmacological doses of melatonin decrease motility and increase colonic transit time (CTT). In contrast, melatonin in lower concentrations increases motility and decrease CTT (24, 113, 116-118).

EC cells also produce serotonin and a recent review highlights the complexity of its role in regulating GI motility (119). Increased serotonin signaling has been shown in the gut in patients with IBS, including increased levels of serotonin in plasma (120), and may contribute to the changed motility and sensation in IBS (121). At physiological levels, melatonin most likely acts as an antagonist of serotonin in regulating gut motility (118).

Irritable bowel syndrome and psychiatric disease

IBS is a functional bowel disorder whose most common symptoms entail abdominal pain and cramping, bloating and swelling of the stomach, altered stool consistency and an urgent need to have a bowel movement. IBS patients, compared with healthy controls, often exhibit greater postprandial abdominal pain, discomfort, urge and greater colonic motility (gastro-colic reflex), as well as an increased stress response and visceral hypersensitivity (122).

There are three main subtypes of IBS: IBS with constipation (IBS-C), IBS with diarrhea (IBS-D) and mixed IBS (IBS-M). Patients who present with symptoms that do not fit into these categories are said to have IBS unclassified (IBS-U) (123, 124).

Low-grade inflammation and disturbances in the brain-gut axis that affect afferent signaling and central processing of nociceptive signals have been proposed to play a role in the pathogenesis and pathophysiology of IBS (125-127). Predisposing factors (e.g., female sex, vulnerability to diarrhea under stress, illness anxiety and somatic symptom burden) and GI infection were found to predict the development of IBS (128). Sex hormones have been suggested to be an important underlying mechanism in sex differences (113, 129, 130).

Patients with IBS have a high prevalence of psychiatric comorbidity, predominantly major depression and anxiety (131). Such comorbidity may be associated with increased symptom severity in patients with IBS (132). Conversely, patients in remission from depression may have no more IBS symptoms than controls (133). Sleep disorders are also prevalent in IBS patients (134-138), possibly mediated by alterations in tryptophan metabolism (134-137).

Although causality has been difficult to establish, there is evidence that in some patients functional GI symptoms arise first and that mood disorders develop later, suggesting that primary gut disturbances might be the underlying driver of the mood disorder in at least some patients (139). It has been

proposed that the comorbidity between depression and IBS could be explained by immune-inflammatory and gut-brain pathways, including oxidative and nitrosative stress as well as tryptophan catabolites (140).

Studies of melatonin administration in patients with IBS have reported ameliorated abdominal pain and reduced rectal pain threshold. These same studies also reported improvements in overall IBS scores and quality of life when melatonin was given orally in the evening (141-143). However, no improvement in sleep disturbances was seen in IBS patients with melatonin treatment (142, 143). Oral melatonin in the dose of 3 mg per night significantly increased CTT in both healthy persons and patients with IBS (144).

Söderqvist and colleagues described the expression of melatonin in EC cells in both normal human GI tract (18) and in tumors derived from these cells (145). Patients with high tumor expression of melatonin reported less diarrhea and high daytime plasma levels of melatonin were associated with nausea (145). Considering the known actions on GI motility, high local accumulation of melatonin in the GI tract could be expected to dampen gut motor activity.

Hepatitis C and treatment with interferon alpha

Hepatitis C virus (HCV) infection is an important cause of chronic liver disease worldwide, with an estimated 185 million people infected and is among the leading causes of end-stage hepatic disease and hepatocellular cancer (146). Until 2011, the most common treatment for HCV was IFN- α and ribavirin (RBV), which are nonspecific immune boosters (147, 148). The side effects have largely been attributed to IFN- α . A major disadvantage of this treatment is the frequent side effects of depressive symptoms experienced by 30-70% of treated patients, with 15-45% developing MDD (149). The high variation in reported incidence rates may be due to variations in case definition across studies with most of them not providing a proper assessment of MDD at baseline and follow-up with validated structured diagnostic interviews (150, 151). When examined with structured diagnostics, about one third of the patients treated with IFN- α develop a major depressive episode (152). With this rather high incidence rate of depression, IFN- α treatment has been used as a model to study inflammation and depressive symptoms.

Several studies have examined pathways underpinning IFN- α -induced depression. From this work, we know that various pathways have been implicated, including an increased secretion of pro-inflammatory cytokines, serotonergic effects, induction of the kynurenine pathway and aberrations in the hypothalamic-pituitary-adrenal (HPA) axis feedback (151, 153-156). These findings have led to the proposal that IFN- α -related depression could constitute a model of cytokine-induced depression, with relevance for de-

pression not related to such treatment (157). Nevertheless, several studies failed to ascertain a diagnosis of a major depressive episode at baseline and endpoint, which imposes a significant limitation to the interpretation of the findings (150). Patients who develop IFN- α -induced depression have an increased biological sensitivity to IFN- α , as shown by larger gene expression changes and specific signatures both as predictors and as correlates (158).

Psychiatric morbidity in patients with HCV infection is elevated and otherwise eligible patients have frequently not received treatment because of fear of an exacerbation of psychiatric symptoms (149, 159-161). In a recent study of patients considered for HCV treatment, the prevalence of a lifetime psychiatric diagnosis was 88% while 54% of patients had a current psychiatric diagnosis (162). In the same material, the prevalence of drug use in the last year was 65% (163). Psychiatric side effects have also compromised adherence, which is critical for the success of HCV therapy (164, 165).

It is important to note that HCV infection may in itself contribute to psychiatric symptoms by inflammatory routes, direct brain neurotoxicity, metabolic and neurotransmitter pathway derangement and immune-mediated responses (166-168).

Hepatitis C and new interferon-free treatment

The arrival of direct-acting antiviral agents (DAAs) has drastically changed HCV treatment. This new treatment has increased the likelihood of cure, which is referred to as “sustained virological response”, with a shorter duration of treatment (169, 170). The current generation of DAAs, including daclatasvir (DCV), sofosbuvir (SOF), simeprevir (SIM) and ledipasvir (LDV) are used without interferon to cure HCV infection (171, 172).

Although the side effect profile of DAAs compared with previous HCV medications is less severe (173), few studies have specifically addressed psychiatric symptoms in DAA treatment (164, 174). More data are needed to assess patient-reported outcomes and adherence of HCV patients in clinical practice (169, 175).

Aims

The overall aim of this thesis was to study the relationship between melatonin, inflammation and depression. The specific aims of each paper are listed below.

- I. To investigate the relationship between daytime salivary melatonin levels and the severity of depressive symptoms.
- II. To study associations between daytime salivary melatonin levels and GI symptoms.
- III. To examine the interaction between daytime salivary melatonin levels and levels of a large panel of inflammatory markers.
- IV. To investigate whether patients with HCV infection, who have an elevated psychiatric morbidity, could successfully undergo treatment with new interferon-free medication without any psychiatric side effects.

Methods

Design

Studies I, II and III were cross-sectional observational studies. A sub-population in study I was followed-up after 6 months. Study IV was a prospective observational cohort study with a longitudinal design and repeated measures.

Setting

Studies I, II and III

The material and data used in papers I, II and III originated from the Uppsala Psychiatric Patient Samples (UPP) cohort, a project designed to collect biological material from patients seeking psychiatric care at the Section for Affective Disorders at Uppsala University Hospital.

All data were collected from patients seeking care between 2012 and 2014 at a psychiatric outpatient clinic for young adults (Swedish: Psykiatrimottagning för unga vuxna). This outpatient clinic provides care for patients aged 18-25 years, with mainly mood and anxiety disorders but also personality disorders and comorbid neuropsychiatric disorders.

Study IV

Patients were recruited at the outpatient clinic for infectious diseases, Uppsala University Hospital between June 2014 and April 2015.

Patients with hepatitis C, but without malignancy, were eligible for DAA treatment. The choice of treatment regimen was based on Swedish national recommendations by The Swedish Medical Products Agency at the time of treatment (176). All patients followed treatment as usual.

Study populations

Paper I

Totally, 722 consecutive patients were asked to participate in the UPP study. Of these 722 patients, 300 (42%) agreed to participate. Of these 300 patients, 125 (42%) completed saliva sampling. Patients that met the criteria for any psychiatric diagnosis according to the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV) were included. Six patients were excluded because they did not fulfill criteria for any DSM-IV Axis 1 diagnosis. In all, 119 (40%) patients with a DSM-IV Axis 1 disorder completed saliva sampling and were enrolled in study I (Figure 4).

Paper II

This study used a subgroup of the patient sample described in study I. The subgroup consisted of 96 (of 119) patients for which saliva samples and data on GI symptoms were available (Figure 4).

Paper III

The study population for study III was the same as for study I, except that blood sampling was missing or incomplete for 11 patients. Thus, the final sample for study IV comprised 108 (91%) patients with saliva and serum blood samples (Figure 4).

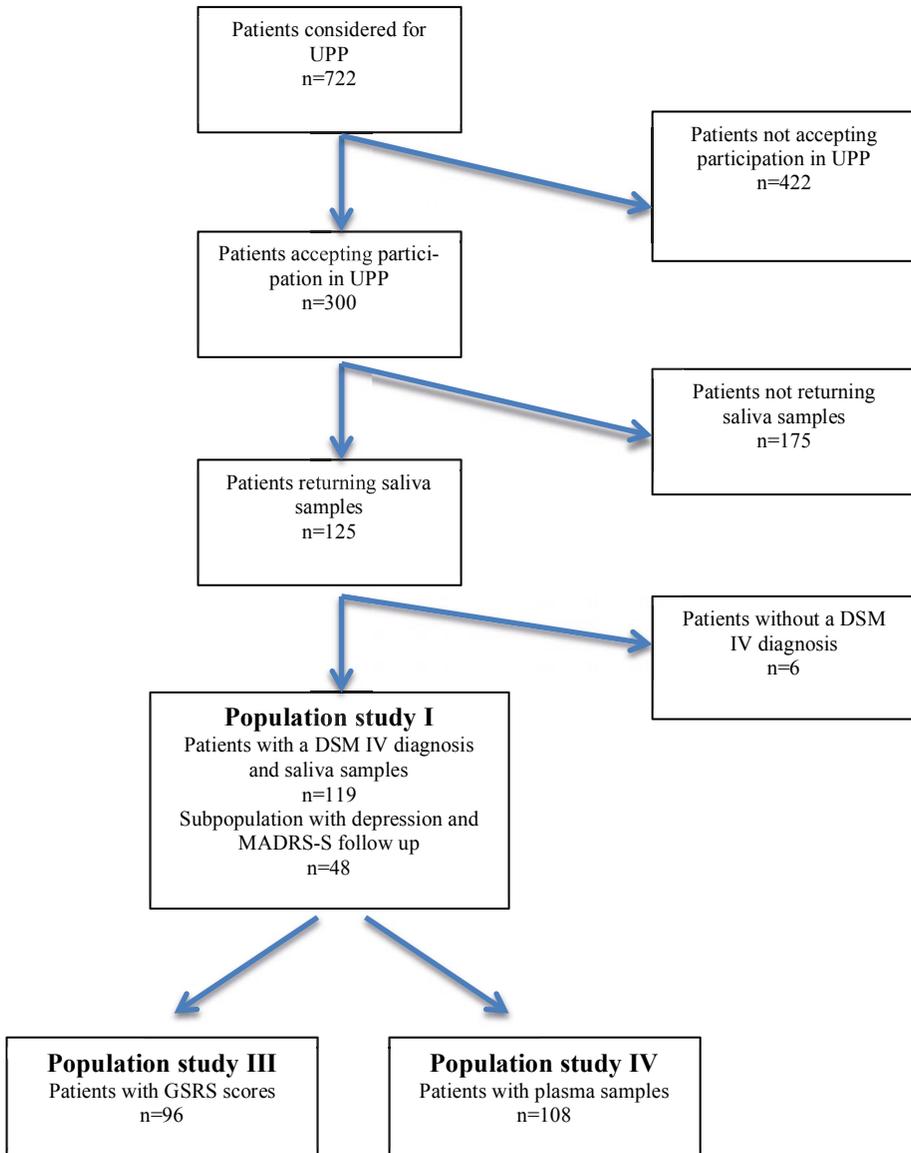


Figure 4. Flowchart for inclusion of patients - papers I, II and III

Paper IV

Sixty-three consecutive patients were initially considered for inclusion. However, 11 were excluded because of social and/or cognitive reasons. Of the 52 eligible patients, 19 (37%) accepted and were included in the study. Two of those patients declined to start the study before the first visit. Thus, the final sample was composed of 17/52 patients (33%) (Figure 5).

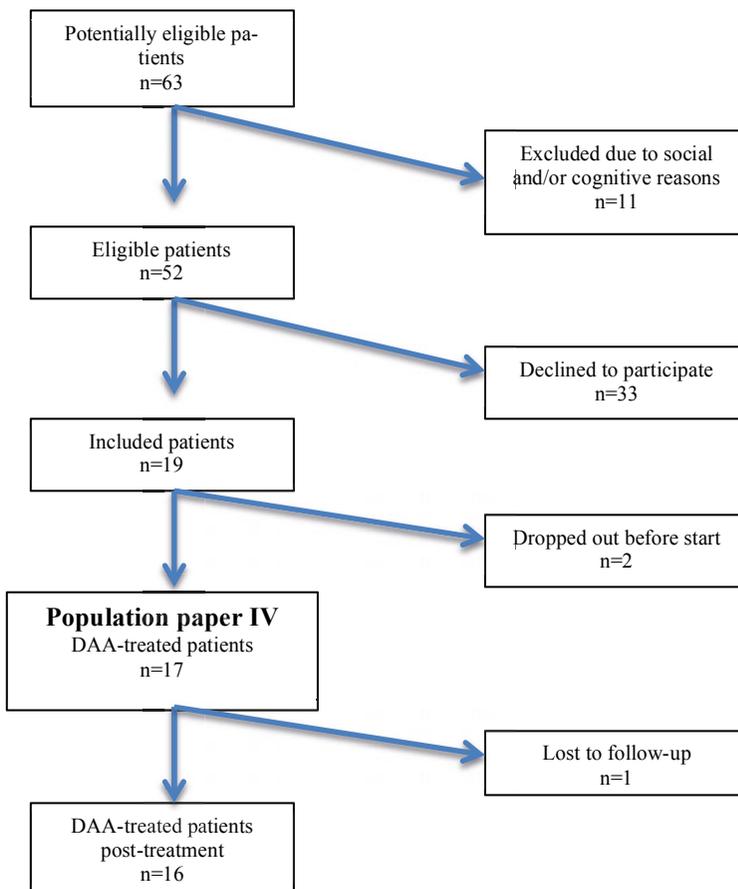


Figure 5: Flowchart for inclusion of patients for paper IV.

Psychiatric assessment and outcome measures/endpoints

Papers I, II and III

Assessment of psychiatric diagnoses were based on the Swedish version of the M.I.N.I. International Neuropsychiatric Interview (M.I.N.I. 6.0) (177), which is a structured diagnostic interview, or the Structural Clinical Interview for DSM IV axis I disorders (SCID-I) (178), which is a semi-structured diagnostic interview. Trained doctors or psychologists performed all interviews.

Self-assessment

Papers I, II and III

Montgomery-Åsberg Depression Rating Scale

Depressive symptoms were measured using the self-rating version of the Montgomery-Åsberg Depression Rating Scale (MADRS-S) (179). The MADRS-S contains nine questions rated on a six-point Likert-like scale from 0 to 6, with 0 indicating “normal/symptom absent” and 6 indicating “severe symptom”. The overall score ranges from 0-54. It has been shown to be a reliable and sensitive self-report tool for depressive symptoms.

For patients with a diagnosis of depression in study I, MADRS-S assessment at clinical follow-up (i.e. 4 to 8 months after baseline) was retrieved when available in the medical records.

The Gastrointestinal Symptom Rating Scale-IBS

GI symptoms were measured with The Gastrointestinal Symptom Rating Scale-IBS (GSRS-IBS) (study II). The GSRS-IBS is a validated self-assessment instrument used to assess GI symptoms of IBS. Total score can range from 13-91 (180).

Physical examination

Physical examination included the measurement of body mass index (BMI) using the formula $BMI = kg/m^2$.

Paper IV

At baseline, patients were assessed for past and current psychiatric morbidity in a clinical interview that included the SCID-I-CV. Previous HCV treatment and psychiatric side effects were also addressed. A trained psychiatrist (the current author) performed all the interviews. To assess the presence of de-

pressive episodes according to the DSM-IV, patients were interviewed using module A of the SCID-I-CV at every following visit.

Self-assessment

Depressive symptoms were measured using the MADRS-S.

Pittsburgh Sleep Quality Index

Sleep quality was measured with the Pittsburgh Sleep Quality Index (PSQI). The PSQI is a validated self-rated questionnaire to assess sleep quality and disturbances over a 1-month time interval. The total score can range from 0-21, where lower scores denote a healthier sleep quality (181). At every visit, patients filled in self-assessment questionnaires (MADRS-S and PSQI).

Alcohol Use Disorders Identification Test and Drug Use Disorders Identification Test

To screen for alcohol and drug use the Alcohol Use Disorders Identification Test (AUDIT) (182) and Drug Use Disorders Identification Test (DUDIT) (183) were completed at baseline, 12 weeks after baseline and 12 weeks post-treatment.

Biological assessments and endpoints

Saliva collection

Saliva samples were collected by the patients at home at six time points during one day: when waking up, 30 min after waking up but before breakfast, at 11.00 h, 30 min after lunch, at 22.00 h and just before going to bed. Time points were chosen to capture salivary melatonin variation during waking hours and after meals in addition to the expected rise in melatonin levels before sleep.

Analysis of melatonin in saliva – papers I, II and III

Melatonin in saliva was measured using the competitive Enzyme-linked immunosorbent assay (ELISA) (Direct Salivary Melatonin Elisa EK-DSM. Bühlmann Laboratories AG.Schönenbuch. Switzerland). Analyses were performed at the Department of Clinical Chemistry at Uppsala University Hospital.

Analysis of inflammatory markers in plasma - paper III

For measurement of inflammatory markers in plasma, a different antibody-based method was used: proximity extension assay (PEA). In short, PEA

technology allows amplification and quantification of antibody-coupled, proximity-dependent DNA templates, reflecting relative protein levels in the sample (184). The relative levels of 91 inflammatory markers were analyzed in 108 plasma samples from the study cohort using the Proseek Multiplex Inflammation panel (Olink Bioscience, Sweden). The preset inflammatory panel initially included 92 proteins. However, the manufacturer excluded one protein (Beta-nerve growth factor). The assay can be used to compare relative protein values between groups but is not an absolute quantification. All samples were analyzed with the same batch of reagents at the Clinical Biomarker Facility at the SciLife Lab in Uppsala. DNA amplification and quantification were carried out using the BioMark™ HD real-time polymerase chain reaction (PCR) platform (Fluidigm, South San Francisco, CA, USA) (Figure 6).

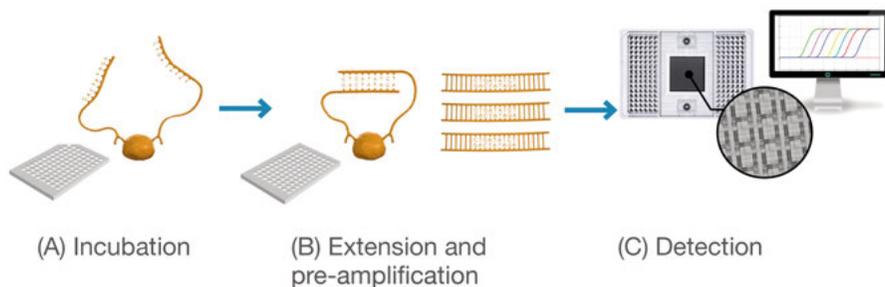


Figure 6: This figure shows the three main steps for the proximity extension assay (PEA) method.

Picture provided by Olink

- A - Incubation: Antibodies with DNA-tag probes bind to the target proteins
- B - Extension and pre-amplification: If both the A and B probes have bound to the protein, they can in this PCR-based step hybridize and become amplified.
- C – Detection: In this read out step, the quantitative PCR (qPCR) technique is used to obtain Ct data, which is then calculated to normalized protein values (NPX).

Validation with the quantitative method - Meso Scale

As a validation step, two of the plasma proteins found to be significantly associated with melatonin levels after correction for multiple analysis were quantified using an electrochemiluminescence sandwich immunoassay from the Meso Scale Discovery (Rockville, MD, USA) multiplex platform.

Hepatitis C virus RNA - paper IV

HCV RNA was analyzed in serum at each time point. All analyses were conducted at the Department of Clinical Chemistry at Uppsala University Hospital. A negative test for HCV RNA at two post-treatment visits was considered a sustained viral response (SVR). SVR is the definition of eradication of the HCV, i.e. HCV eradication.

Statistics

Paper I

Spearman's test (ρ) was applied for pairwise correlations between melatonin levels for each time point to total MADRS-S scores.

A generalized linear model analysis for quartiles of melatonin in relation to baseline MADRS-S scores was performed, first without and then with the inclusion of potential confounding factors: sex, BMI, anti-depressive medication, use of oral contraceptives and the influence of summer or winter season.

For those patients with depression and where the MADRS-S at follow-up was available, an odds ratio (OR) for response was calculated in a logistic regression model. Response was defined as $\geq 50\%$ reduction in the MADRS-S between baseline and follow-up. To control for the influence of baseline depressive symptoms the model was used first without and then with baseline MADRS-S. A p -value of < 0.05 was considered statistically significant for all analyses.

Paper II

Saliva melatonin values at 30 min after waking up, at 11:00 and after lunch were selected for analysis of GSRS-IBS scores in a generalized linear model. Potential confounding factors were included in the analyses: sex, BMI, anti-depressive medication and the use of oral contraception. A p -value of < 0.05 was considered statistically significant and the Bonferroni method was applied to correct for multiple comparisons (reported as q -values).

Paper III

The association between protein level (NPX) and \log_{10} (melatonin) was determined using linear regression with the protein value (NPX) as the dependent variable and melatonin and relevant covariates as independent variables. The association was determined after adjustment for covariates using a likelihood ratio test. The covariates were sex, BMI, use of antidepressant medication and use of oral contraceptives. Of the 91 proteins on the PEA

array, we excluded those with more than 50% of the values below the limit of detection. This exclusion criterion led to the elimination of 19 markers, leaving 72 markers for further analysis. All tests were corrected for multiple testing by applying Bonferroni correction (significance threshold $\alpha=0.05/72=0.0007$).

Paper IV

We used Friedman's test to calculate differences in the MADRS-S, PSQI, AUDIT, DUDIT and GSRS over time. The Spearman's rank correlation test was used to assess the relationship between depressive symptoms and HCV viral load at baseline. In a post-hoc analysis Wilcoxon's test was applied to compare baseline MADRS-S with post-treatment MADRS-S.

Summary of results

Melatonin levels and depressive symptoms (paper I)

Bedtime melatonin was inversely correlated with the MADRS-S total score ($\rho=-0.28$, $p<0.05$). In a generalized linear model patients with bedtime melatonin levels in the two lowest quartiles were at higher risk for an elevated MADRS-S score when compared with patients within the highest melatonin quartile ($p<0.01$). The elevated risk remained after controlling for BMI, sex, use of antidepressant medication, use of oral contraceptives and season ($p<0.01$).

Concerning patients with current depression ($n=68$), follow-up of the MADRS-S was available in the medical records of 27 (40%) patients. There was a positive correlation between baseline melatonin and the reduction in MADRS-S scores between baseline and follow-up ($\rho=0.39$, $p<0.05$), i.e. higher bedtime melatonin levels were related to a larger reduction in total MADRS-S scores. Patients with depression and low bedtime melatonin values had lower odds of response. The OR for response was 4.4 (95% confidence interval [95%CI] 1.06–18.43, $p<0.05$), i.e. with each log increase in bedtime melatonin, the likelihood for reduction of MADRS-S with at least 50% between baseline and follow-up increased 4.4 times. When controlling for baseline MADRS-S, the OR was 4.3 (95%CI 0.98–18.97, $p=0.05$).

Melatonin levels and GI symptoms (paper II)

In the generalized linear model melatonin values after lunch correlated with the total GSRS-IBS score ($p=0.015$). This correlation remained after correction for multiple testing ($q=0.045$). The GI pain ($p=0.047$) and bloating ($p=0.033$) subscales were those with the strongest association to postprandial melatonin.

Melatonin levels and inflammatory markers (paper III)

After correction for multiple testing, there were no significant correlations between bedtime melatonin and inflammatory markers in the Proseek Multi-

plex Inflammation panel (Figures 7 and 8). Thus, the primary hypothesis that melatonin levels in the evening would correlate with levels of inflammatory markers was not confirmed.

For daytime melatonin values and inflammatory markers, postprandial melatonin was positively correlated with vascular endothelial growth factor A (VEGF-A) before ($p=5.8e-6$) and after ($p_{adj}=4.1e^{-4}$) correction for multiple testing, monocyte chemoattractant protein-1 (MCP-1) ($p=4.2e^{-4}$, $p_{adj}=0.030$) and monocyte inflammatory protein-1 α (MIP-1 α) ($p=6.5e^{-4}$, $p_{adj}=0.047$) (Figures 7 and 8).

Melatonin at 11:00 h was positively correlated with cluster of differentiation 5 (CD5) ($p=4.2e^{-4}$, $p_{adj}=0.030$) (Figures 7 and 8).

Quantitative validation showed that plate-normalized NPX values from PEA and log2-transformed meso scale values (pg/ml) were correlated with each other: VEGF-A ($r=0.56$, $p<0.001$) and MCP-1 ($r=0.60$, $p<0.001$).

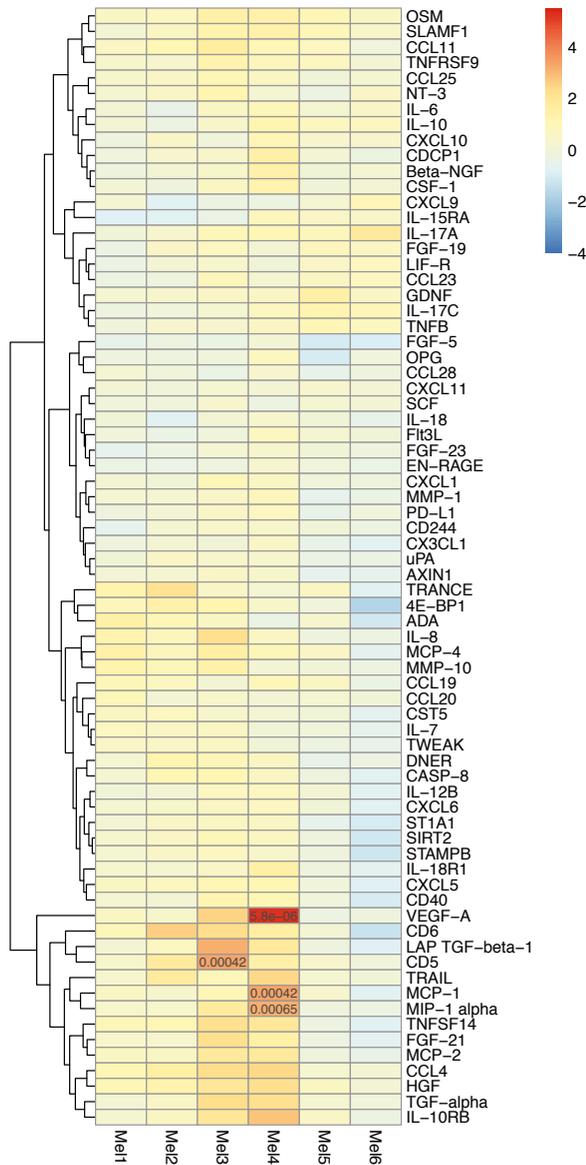


Figure 7: Heat map summarizing the associations between protein (Olink) data and melatonin levels. The heat map is based on all samples and colored according to $-\text{sign}(\beta) \times \log_{10}(p)$, where β is the \log_{10} -melatonin coefficient in the linear regression model predicting protein level (adjusted for sex, BMI, antidepressant medication and anticonception) and p is the LRT p-value. Hence, all negative associations are colored blue and all positive associations red. The p-values are printed for all associations significant at the 0.05 level after Bonferroni correction.

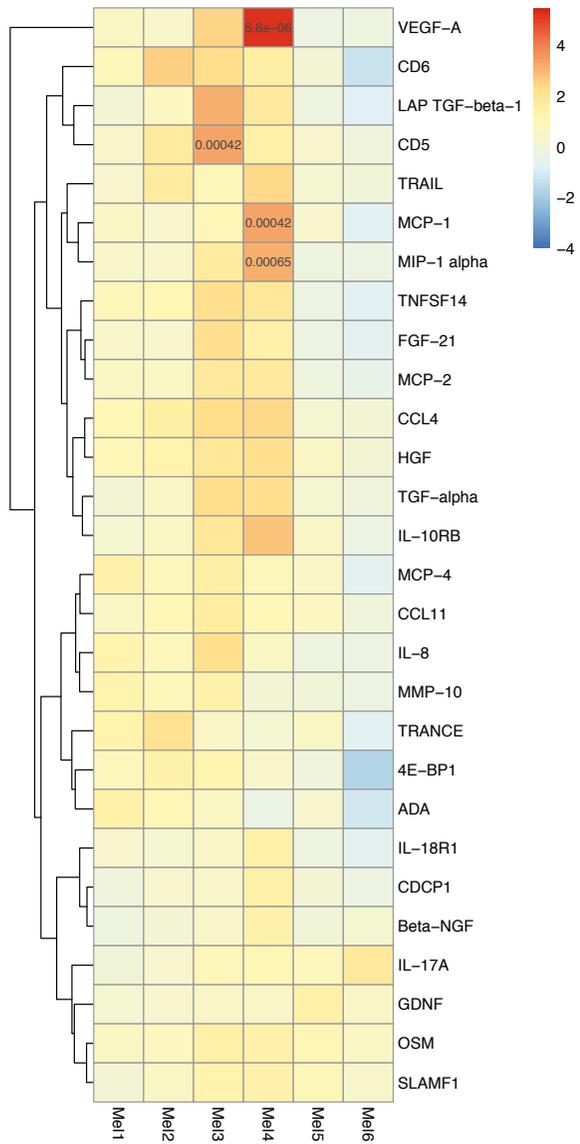


Figure 8: Heat map showing only proteins with any association to at least one melatonin value with a $p < 0.05$ before correction for multiple testing. Mel1: Melatonin when waking up; Mel2: Melatonin 30 min after waking up; Mel3: Melatonin at 11:00 h; Mel4: Melatonin 30 min after lunch; Mel5: Melatonin at 22:00 h; Mel6: Melatonin before going to bed.

Interferon-free treatment for hepatitis C (paper IV)

At baseline, 15/17 (88%) patients had a history of any psychiatric diagnosis while 11 (65%) had a history of substance abuse or dependence. Eleven patients (65%) had previously been treated with interferon and six (35%) of those had experienced significant psychiatric side effects. There was no correlation between HCV viral load and depressive symptoms at baseline. Adherence to treatment was estimated to 95% and the SVR was 88%.

Depressive symptoms did not increase during DAA treatment. On the contrary, depressive symptoms were significantly lower at 12 weeks post-treatment compared with baseline (MADRS-S=8.3 vs. MADRS-S=10.7). This observation held when excluding patients taking antidepressant medication (n=3) (Figure 9).

Sleep quality did not significantly change during treatment: PSQI=7.7 at baseline vs. 7.6 at 12 weeks after treatment ended (Figure 9).

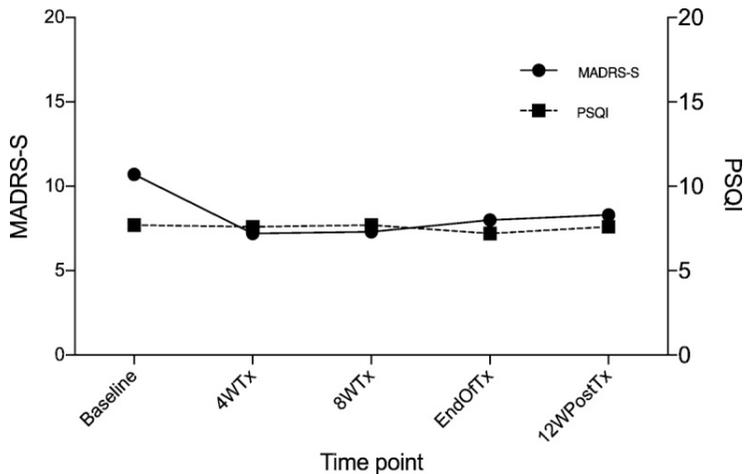


Figure 9: Depressive symptoms (MADRS-S) and sleep disturbance (PSQI) did not increase during treatment with DAAs. MADRS-S was significantly lower post-treatment.

Discussion

The studies in this thesis sought to investigate different aspects of the relationship between melatonin, inflammation and depression. They have confirmed previous evidence showing a link between bedtime melatonin and depressive symptoms and indicated that melatonin levels at bedtime may have prognostic value in depression. A relationship between levels of post-prandial melatonin and GI symptoms has been demonstrated. An inflammatory PEA platform was shown to capture shifts in markers of inflammation in relation to saliva melatonin and depressive symptoms during treatment with interferon. The PEA platform was then applied to study association between daytime melatonin and several markers of inflammation. Lastly, we demonstrated that real-life patients with hepatitis C could successfully undergo treatment with new interferon-free medication, without increased depressive symptoms or sleep disturbances.

Bedtime melatonin and severity of depressive symptoms

In paper I, we observed a negative correlation between evening melatonin levels and dimensional measures of depressive symptoms in young adult patients seeking psychiatric care. This relationship was independent of a diagnosis of current depressive episode. Previous studies have mainly focused on differences at the group level during nighttime between patients with depression and healthy controls.

Depressed patients with low levels of bedtime melatonin at baseline were less likely to show improvement in the overall MADRS-S score at follow-up, suggesting that melatonin values at bedtime may have prognostic implications in depression.

The results in paper I are in line with studies showing a correlation between melatonin levels and depression severity (32, 39, 42) and corroborate the proposed relationship between evening melatonin levels and depressive symptoms (29).

Our tentative explanation of the findings in paper I is that inflammatory routes associated with depression (i.e. pro-inflammatory cytokines) are in-

volved in mediating a relative shut down in the pineal production of melatonin (likely via the NF- κ B-pathway).

Daytime melatonin and GI-symptoms

The results from paper II confirmed the hypothesis that daytime melatonin levels are correlated to GI symptoms as measured by the GSRS-IBS. The mechanism through which higher levels of melatonin are linked to more symptoms of bloating and pain could involve effects of melatonin on GI motility, with decreased motility leading to bloating and pain. These results are consistent with research suggesting that melatonin inhibits contraction and decrease motility (109, 118), and correlations between GI symptoms and melatonin production in patients with neuroendocrine tumors (145). An increase in EC cells, a potential source of daytime melatonin, has been described in patients with IBS (185). Speculatively, this elevation in EC cells could increase melatonin levels, at least in a subpopulation of patients.

Daytime melatonin and inflammatory markers

The main finding in paper III was that bedtime melatonin levels were not related to inflammatory markers in blood. Thus, our primary hypothesis was rejected. Daytime saliva levels of melatonin, however, were positively correlated to several markers of inflammation. Specifically, after correction for multiple testing, daytime postprandial melatonin levels were associated with levels of VEGF-A, MCP-1 and MIP-1 α and melatonin levels at 11:00 were associated with levels of CD5. Elevated postprandial levels of melatonin and the cytokines VEGF-A and MIP-1 α were more common in patients with anxiety disorders, whereas MCP-1 was associated with a diagnosis of depression.

The associations between daytime melatonin levels, inflammatory markers and anxiety disorders in this study are in accordance with those associating VEGF-A, MCP-1 and MIP-1 α with depression and anxiety (186-191). The results further underscore an association between melatonin and the immune system in a clinical context of psychiatric patients.

Results from paper III may suggest that perhaps depression is more related with the shutdown of pineal melatonin, whereas anxiety is more related to melatonin counteracting oxidative stress. This interpretation would be in line with results from a study showing that severity of anxiety - but not depression - is associated with oxidative stress in MDD (192).

The strength of this study concerns the validation of selected cytokines in a system measuring absolute levels. Moreover, confirmation of inflammato-

ry markers previously found related to psychiatric disorders provides support for the validity of the findings and against type 1 errors.

Another possible interpretation for the correlations between VEGF-A, MCP-1, MIP-1 α and postprandial melatonin pertains to glycemic control. Several studies have shown that individuals with poor glycemic control have higher postprandial serum levels of VEGF, MCP-1 and MIP-1 α (193-196). In paper III, we also noted a significant positive association between VEGF-A, MCP-1, MIP-1 α and BMI. In this respect, it can be noted that melatonin has emerged as a hormone important for glucose regulation (197-199). A potential relationship between daytime postprandial levels of melatonin and insulin resistance merits further investigation.

The fact that bedtime melatonin was not related to inflammatory markers (after correction for multiple testing) may also be due to low statistical power. Had the study population been larger, perhaps such a relationship could have been detected.

Psychiatric symptoms during interferon-free treatment for hepatitis C

In paper IV, real-world patients with hepatitis C were able to complete DAA treatment successfully without side effects as regards sleep or depressive symptoms. This success occurred despite that a majority of the patients in the study had a history of affective disorder, drug abuse, neuropsychiatric disorder or previous interferon-based treatment. Depressive symptoms were reduced after successful DAA treatment.

Our study confirms previous findings demonstrating that HCV patients with psychiatric comorbidity can be treated with DAAs with good efficacy and without psychiatric side effects. This finding is particularly noteworthy given that these patients have previously often been excluded from HCV treatment.

Interferon-based treatments stimulate production of TNF- α and other pro-inflammatory cytokines, ultimately resulting in depressive symptoms in some patients. DAA-treatment likely has a different effect on inflammatory cytokines (200). This may explain the divergence in side effects of depressive symptoms and sleep disturbance between these types of treatment.

Patients in paper IV had lower scores of depressive symptoms after being cured of hepatitis C. This could be due to both the positive psychological consequences of being free from an otherwise chronic disease or that the viral infection no longer is present to stimulate low grade inflammation and thereby promoting depressive symptoms.

One patient in our cohort was lost to follow-up because of *de novo* hepatocellular cancer (HCC) shortly after completing DAA treatment. Concerns

have been raised of DAAs increasing the risk for HCC (201). However, subsequent studies have not found any evidence to suggest that DAAs promote HCC (202). It has been suggested that the apparent increase in HCC incidence observed in patients with cirrhosis treated with DAAs compared with patients who achieved SVR following an IFN therapy could be explained by patient characteristics and lower screening intensity (203). In conclusion, the concern of DAAs increasing risk for HCC seems to have lost its importance (204).

General comments

Taken together, the results from the studies in this thesis point towards a relationship between melatonin and inflammation. The findings are in line with previous studies suggesting that inflammatory cytokines differentially regulate pineal and extra-pineal melatonin (51-53). This is reflected in the negative association between bedtime melatonin and depressive symptoms, in contrast to the positive association between daytime melatonin and inflammatory markers.

For behavior and symptoms to be altered by peripheral inflammation, there has to be a mechanistic connection between peripheral inflammatory markers and processes in the CNS. It has been proposed that pro-inflammatory cytokines in the periphery can cause increased BBB permeability that allows influx of peripheral blood mononuclear cells to the CNS. Such activity can lead to activation of microglia producing ROS, QUIN and pro-inflammatory cytokines (e.g., MCP-1 and MIP-1 α), which drives psychiatric symptoms such as anxiety (205). This supposition provides a possible link to understanding the association between pro-inflammatory cytokines in the periphery and depression and anxiety symptoms.

The results in these studies may have relevance for several types of disorder related to low-grade inflammation (206-209), i.e. disorders other than depression and anxiety, as other disorders may share the same underlying pathological mechanisms.

We could not determine the source of the measured melatonin from saliva. Theoretically, saliva levels of melatonin could come from several different sources in various proportions, depending on time of sampling and, as proposed in this thesis, depending on inflammatory status. Melatonin in saliva partly reflect those in the GI tract during the daytime (18, 210), and perhaps so to a larger extent after lunch. Melatonin in saliva could also come from the pituitary, although this production is down-regulated during the daytime and even more so during inflammation. Thus, saliva levels in the evening and when waking up are in large part likely to be dependent on melatonin release from the pituitary. Melatonin from other melatonin-producing cells, including immune cells in the periphery, acts foremost locally. There-

fore, the amount of melatonin from these sources reaching the blood stream and saliva is likely minor but more constant during the day.

Methodological considerations and limitations

All the patients in this thesis work have been diagnosed with structured or semi-structured diagnostic interviews. This speaks for the validity of diagnoses in the material presented.

The self-rating scales MADRS-S and GSRS-IBS are used outside their intended scope in this thesis. MADRS-S was designed to follow depressive symptoms in patients diagnosed with depression, whereas we have used the scale as an indicator of depressive symptoms also in patients without a diagnosis of depression. In analogy, we have used the GSRS-IBS to measure GI symptoms in a population without IBS. The motivation for this “irreverent” treatment of these scales is that they are used to cover dimensional symptoms.

The four studies in this thesis contain several limitations. First, as melatonin levels during sleep were outside the scope of this thesis work, nothing could be said about the relationship between melatonin during sleep, symptoms and inflammatory markers. Nor could we assess the timing of melatonin, i.e. if variations in melatonin levels in the evening were due to a phase shift or to differences in absolute levels. In addition, the limited amount of saliva did not allow for serial dilutions for concentrations above 50 ng/L.

One speculative explanation for the fact that we did not see an association between inflammatory markers and bedtime melatonin may be that, while inflammation may cause down-regulation of pineal melatonin, and inflammation at the same time causes up-regulation of melatonin in the periphery, in the evening the result of these mechanisms may counteract each other. Therefore, we can observe the association between melatonin and inflammatory markers during the daytime, but not in the evening. One could speculate that, had we measured melatonin in the middle of the night, we would have found a negative association between inflammatory markers and melatonin.

The inclusion rates in the studies were relatively low. There are several reasons for these low rates. In the studies of young adults, patients with psychiatric conditions - which possibly affected their motivation, energy, drive and ability to concentrate - were asked to join the study and to perform saliva sampling at home and to bring back the samples. It could be that patients with severe affective or neuropsychiatric symptoms were less likely to accept participation or to complete the study. On the other hand, patients with only lighter symptomatology may not be as motivated to participate in a clinical study. Thus, we may have lost participants for two rather opposing conditions: patients who are too ill and those who are lack motivation be-

cause they are only mildly ill. Both these scenarios could have led to a differential selection effect.

We cannot compare the 422 patients who choose not to participate to those who accepted. However, analysis of internal dropouts showed that those who completed saliva sampling (n=119) differed from the total group initially accepting participation (n=300) in sex (85% female in those who completed saliva sampling vs. 75% female in the total group, $p=0.029$) and BMI (24.4 vs. 21.5, $p=0.01$) while total MADRS-S score (23.3 vs. 21.5) and the distribution of diagnoses were not statistically different: depressive episode (57% vs. 50%) and any anxiety disorder (66% vs. 64%).

With reference to the study of patients with hepatitis C, the relatively low inclusion rate could, in part, be explained by an extensive study protocol with repeated measures and by the fact that several other clinical studies were ongoing in the clinic at the same time. However, given the high correlation with larger patient materials, the small patient sample is likely representative of real-life patients considered for hepatitis C treatment.

Blood sampling for inflammatory markers was performed during office hours the day after saliva sampling, but not at a specified time point. This sampling procedure could have introduced the risk of random error, but less likely systematic bias leading to type II errors. Several inflammatory markers have circadian rhythms (211, 212), whereas others (e.g., CRP, TNF- α , IL-8, IL-10 and VEGF-A) show minimal circadian variation (62, 213).

The analyses in studies I, II and III were adjusted for sex, BMI, antidepressant medication and use of oral contraceptives, which is a strength relative to many other studies. However, complete data were not available for other lifestyle factors (e.g., exercise, alcohol use and smoking), which may have influenced levels of inflammatory markers (191). Furthermore, the sample sizes may have limited the power to identify the full extent of the relationship between inflammatory markers and melatonin levels.

Finally, the cross-sectional design of studies I-III cannot provide information on the causal relationship between melatonin and inflammation over the course of a disease.

Epilogue to clinical case

Patient X received antidepressant treatment, was able to complete interferon treatment and has been free from hepatitis C and depression since.

Clinical implications and future perspectives

There are clear links between melatonin, inflammation, anxiety and depressive symptoms. However, clinical practice has not come to the point where patients with depression are categorized based on underlying pathological processes, e.g. regarding inflammation, as a step in order to find the best treatment.

In the future we will probably see a greater degree of stratification of patients according to different biomarkers. Those patients with elevated inflammatory status will theoretically benefit from treatment addressing inflammation. There are several ongoing and planned randomized controlled trials in this field. Some of these studies include adding anti-inflammatory treatment to antidepressants (214), or testing monoclonal antibodies against inflammatory cytokines in patients with depression and low-grade inflammation (215).

Given indications that patients with higher inflammatory variables would likely benefit more from certain antidepressant treatments, e.g. those treatments modulating the dopamine system (216), some authors argue that with our current knowledge, we can already make decisions of treatment strategy based on basic inflammatory parameters (such as CRP), when treating patients with depression (216). With more evidence, i.e. if these results are validated in a clinical setting, strategies of personalized antidepressant treatment could come into routine practice.

It has been argued that targeting immune dysfunction, oxidative stress and circadian rhythm misalignment may lead to disease-modifying drugs that could prevent neurodegeneration rather than simply alleviate symptoms with no curative intent (217). It has also been proposed to identify groups of biomarkers for screening and early detection of depression in high-risk groups (218).

To maximize the output in clinical research, further research should study the same individuals with several measures from different perspectives to gain knowledge on how biological processes are linked to aberrant behavior and symptoms. In this sense, a well characterized patient sample with measures both in the DSM tradition of discrete diagnoses and underlying constructs of psychopathology in the spirit of RDoC are desired. Furthermore, future studies need to employ a longitudinal design with repeated measurements to answer questions of causality and how the relationships among variables evolve over time.

Ultimately, this will not only lead to a fuller appreciation of the underlying biological processes but should also aid clinicians to avail their patients with the advantages of existing and new therapies. All this holds promise for patients with depression who are currently not receiving optimal care.

Ethics

The projects included in the thesis obtained Ethics approval by the Regional Ethics Committee in Uppsala: (Dnr 2012/81, Dnr 2012/81/1, Dnr 2013/219).

The studies were performed in full accordance with the Declaration of Helsinki. All patients received verbal and written information about the studies and provided written informed consent. Filling in repeated questionnaires takes time and can be bothersome. The most invasive test was venous blood puncture. The patient in the clinical case in the introduction has been anonymized.

Conclusions

- Evening levels of melatonin in saliva were negatively correlated with depressive symptoms in young adults seeking psychiatric care. In patients with depression, lower evening levels of melatonin were associated with worse prognosis 6 months after baseline assessment.
- Postprandial levels of melatonin in saliva were positively correlated with GI symptoms of bloating and pain in young adults seeking psychiatric care.
- Postprandial levels of melatonin were positively correlated with inflammatory markers (VEGF-A, MCP-1 and MIP-1 α) in blood. Evening levels of melatonin did not correlate with inflammatory markers. VEGF-A and MCP-1, as well as postprandial levels of melatonin were correlated with anxiety disorders, whereas MIP-1 α was correlated with MDD.
- Patients with hepatitis C underwent treatment with new interferon-free drugs against hepatitis C without substantial psychiatric side effects in terms of sleep or depressive symptoms. On the contrary, depressive symptoms were lower after treatment.

Sammanfattning på svenska

Depression

Depression är en av våra stora folksjukdomar. Det är en av de sjukdomar som bidrar till mest ohälsa i världen. Vidare är risken att drabbas av kroppsliga sjukdomar ökad hos personer med depression. Självmord är den näst vanligaste dödsorsaken i åldersgruppen 15-29 år och den vanligaste underliggande orsaken är depression. Samtidigt är det så, att var tredje patient med depression inte blir frisk med de behandlingar som nu finns tillbuds.

Sannolikt är det så att det vi kallar depression kan uppkomma genom olika mekanismer. Inflammatoriska processer i hjärnan och i resten av kroppen, samband mellan mag-tarmkanalen och hjärnan, och hur hormonet melatonin samverkar med dessa, är mekanismer som studerats. Det är just kopplingen mellan inflammation, melatonin och depression som är ämnet för denna avhandling.

Melatonin

Melatonin bildas dels i tallkottkörteln i en rytm som styrs av dagsljuset via ögonen. Melatonin i sin tur styr sömn och dygnsrytm. Samtidigt verkar det vara så, att melatonin kan bildas av samtliga celler i kroppen. Särskilt mycket melatonin bildas i mitokondrierna – cellernas ”kraftverk”. Detta är sannolikt därför att melatoninnet där hjälper till att neutralisera fria syreradikaler som bildas i stor omfattning under energiproduktionen, och som annars riskerar att skada och förstöra cellerna. Stora mängder melatonin finns även i mag-tarmkanalen och bidrar där till att upprätthålla tätheten i tarmens väggar. Melatonin kan även påverka rörligheten i mag-tarmkanalen.

Inflammation verkar kunna leda till depression via olika mekanismer. Fler av dessa mekanismer inbegriper budbärarmolekyler - cytokiner - som gör att olika delar av kroppen kan kommunicera med varandra.

Hepatit C

Hepatit C är en virussjukdom som drabbar levern. Patienter med hepatit C har tills nyligen behandlats med interferon alpha - en slags cytokin som stimulerar immunförsvaret att döda hepatitviruset. Som biverkan har cirka var tredje patient som behandlats med interferon utvecklat depression. Därför har interferonbehandling av hepatit C använts som modell för inflammationsorsakad depression.

Nya mediciner mot hepatit C, vilka inte innehåller interferon, har kommit ut på marknaden. Huruvida dessa nya mediciner har biverkningar i form av depressiva besvär är däremot otillräckligt studerat.

Syfte

Avhandlingen har haft fyra syften:

- I. Att undersöka kopplingen mellan melatonin i saliv på kvällen och depressiva besvär.
- II. Att undersöka kopplingen mellan melatonin i saliv dagtid och besvär från mag-tarmkanalen.
- III. Att undersöka sambandet mellan melatonin i saliv dagtid och flera olika inflammatoriska markörer (varav flera cytokiner).
- IV. Att se huruvida patienter med hepatit C, vilka ofta har psykiska besvär sedan tidigare, kan genomgå behandling med nya mediciner, som inte innehåller interferon, utan att få psykiatriska biverkningar.

Metod

Unga vuxna patienter mellan 18-25 år undersöktes i uppsats I, II och III. Melatonin i saliv mättes med en teknik som kallas ELISA. Depressiva besvär mättes med självskattningsskalan MADRS-S. Inflammatoriska markörer i blod mättes med PEA.

I uppsats IV undersöktes patienter med hepatit C, dels innan, dels under tre månaders behandling med nya mediciner mot hepatit C, och dels efter behandlingen. Förutom depressiva besvär mättes även grad av sömnstörning och huruvida patienterna blivit fria från viruset i blodet, d.v.s. friska från hepatit C.

Resultat och slutsats

- I. Melatonin i saliv på kvällen var omvänt kopplat till depressiva besvär, d.v.s. ju lägre melatonin på kvällen, desto mer depressiva besvär. Hos patienter med depression var melatoninnivåer på kvällen även kopplat till sämre prognos.
- II. Melatonin i saliv efter lunch var kopplat till mag-tarmsymptom som uppkördhet och smärta.
- III. Melatonin efter lunch var kopplat till flera inflammationsmarkörer i panelen (däribland VEGF-A, MCP-1 och MIP-1 α). Melatonin på kvällen, däremot, var inte kopplat till några av inflammationsmarkörerna. De inflammatoriska markörerna VEGF-A och MCP-1 var, liksom melatonin efter lunch, kopplade till ångestsjukdomar, medan markören MIP-1 α visade sig överrepresenterad hos dem med depression.
- IV. Patienter med hepatit C kunde genomgå behandling med de nya medicinerna, utan att få biverkningar i form ökade depressiva besvär eller sömnstörning. Tvärtom hade patienterna något minskade depressiva besvär efter genomgången behandling.

Sammantaget visar dessa studier att det finns kopplingar mellan inflammation, melatonin och depression. I framtiden kommer kanske markörer för inflammation kunna användas för att hitta subgrupper av patienter som svarar särskilt bra på vissa behandlingar mot depression. Möjligen kommer även nya mediciner som riktar sig mot inflammation kunna hjälpa patienter som lider av depression och där det finns en inflammatorisk komponent.

Patienter med hepatit C har tidigare ibland undanhållits behandling med interferon, eftersom man varit rädd för psykiatriska biverkningar av denna behandling. Fynden i denna avhandling stärker att patienter med hepatit C framgångsrikt kan behandlas med nya interferonfria mediciner utan att få psykiatriska biverkningar.

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