Melatonin and its receptors in the normal human gastrointestinal tract, pancreas and in small intestinal neuroendocrine tumours

Licentiate Thesis

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2017
Abstract


Melatonin, “the hormone of darkness” is well known to regulate sleep and circadian rhythm. However, melatonin is also present in numerous peripheral tissues and the number of actions assigned to this neurohormone is growing steadily. Based on animal studies, it has been proposed that gastrointestinal melatonin is produced in enterochromaffin cells.

The aims were to characterise the expression of melatonin and its receptors MT₁ and MT₂ in normal human gastrointestinal tract and pancreas as well as in tumours derived from enterochromaffin cells, small intestinal neuroendocrine tumours (SI-NET), using immunohistochemistry. Melatonin and receptor expression was furthermore compared to clinical symptoms, tumour prognostic factors and treatment response.

In enterochromaffin cells from normal gastrointestinal tissue and in SI-NETs a strong immunoreactivity (IR) for melatonin and MT₂ was found, while MT₁ IR was low or absent. Melatonin, MT₁ and MT₂ IR was also seen in the large intestinal epithelium of normal gastrointestinal tract and in pancreatic islets, although the expression of MT₁ in pancreatic tissue varied. Analyses of mRNA data confirmed the expression of the enzymes needed for melatonin synthesis as well as MT₁ and MT₂ in small intestine and pancreas.

The intensity of melatonin IR in SI-NETs correlated to lower proliferation index and less symptoms of diarrhoea, which is well in line with the proposed actions of melatonin described in animal studies. The intensity of MT₂ IR was generally lower in metastases than in primary tumours. Plasma levels of melatonin in patients with SI-NETs and disease stabilisation/remission were reduced after treatment and higher levels were associated with nausea.

In conclusion, melatonin and its receptors are present in the normal human gastrointestinal tract, pancreas and in SI-NETs. Melatonin IR intensity in tumours correlated significantly to less diarrhoea and to lower proliferation index. Plasma levels of melatonin in patients with SI-NETs were reduced with treatment response, indicating a possible tumour-derived origin of circulating melatonin levels.

These results are in agreement with the suggested actions of melatonin on gastrointestinal motility and tumour growth.
To my family
List of Papers

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


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Abstract

List of Papers

Abbreviations

1. Introduction
   1.1 What is a hormone?
   1.2 Melatonin, general aspects
   1.3 Melatonin receptors, MT₁ and MT₂
   1.4 Melatonin and the brain
   1.5 Melatonin in the gastrointestinal tract
   1.6 Other regulatory functions
      1.6.1 Immunomodulatory functions
      1.6.2 Glucose homeostasis
   1.7 Melatonin and cancer
   1.8 Small intestinal neuroendocrine tumours

2. Aims of the studies

3. Materials and methods
   3.1 Patient samples
      3.1.1 Study I
      3.1.2 Study II
   3.2 Immunohistochemistry
      3.2.1 Study I. Histological assessment
      3.2.2 Study II. Histological assessment
   3.3 Microarray analysis
   3.4 Hormone analyses
   3.5 Statistical analyses

4. Results and discussion
   4.1 Expression of melatonin and its receptors in normal human gastroenteropancreatic tract and in SI-NETs
   4.2 Melatonin and its receptors in human pancreas
   4.3 Melatonin and its receptors in patients with SI-NETs, anti-tumourigenic effects
   4.4 Plasma levels of melatonin in patients with SI-NETs and the brain

5. Methodological considerations

6. Conclusions

7. Future studies

8. Acknowledgements

9. References
<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AANAT</td>
<td>Arylalkylamine N-acetyltransferase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CgA</td>
<td>Chromogranin A</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P</td>
</tr>
<tr>
<td>EC</td>
<td>Enterochromaffin</td>
</tr>
<tr>
<td>GEO</td>
<td>Gene Expression Omnibus</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>IBS</td>
<td>Irritable bowel syndrome</td>
</tr>
<tr>
<td>IF</td>
<td>Immunoflourescence</td>
</tr>
<tr>
<td>HIOMT</td>
<td>Hydroxyindole-O-methyl transferase</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IR</td>
<td>Immunoreactivity</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic acid</td>
</tr>
<tr>
<td>MT1</td>
<td>Melatonin receptor type 1</td>
</tr>
<tr>
<td>MT2</td>
<td>Melatonin receptor type 2</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>ROR/RZR</td>
<td>Retinoid orphan receptors/retinoid Z receptors</td>
</tr>
<tr>
<td>SCN</td>
<td>Suprachiasmatic nucleus</td>
</tr>
<tr>
<td>SI-NET</td>
<td>Small intestinal neuroendocrine tumour</td>
</tr>
</tbody>
</table>
1. Introduction

1.1 What is a hormone?

Hormones are chemical messengers that help the organs and cells to communicate. There are many different types of hormones: proteins, peptides, amino acids, steroids, fatty acid derivatives, ions and gases. All nucleated cells have the genetic information required to produce hormones (1).

The target receptor, the type of cell that expresses it and its location, determines the effect of the hormone and the same hormone can exert vastly different effects in different tissues. Ligand binding activates the receptor, which in turn sets off a cascade of intracellular signalling pathways resulting in changed behaviour of the cell.

The terms autocrine, paracrine and endocrine signalling, describe three different levels of signalling, based on where the target cell is located. Autocrine signalling implies that the hormone sends its message to the same cell from which it was secreted, for example cancer cells producing growth factors, that stimulate their own survival and proliferation (2). In paracrine signalling the receiver of the hormonal message are neighbouring cells from where the hormone was secreted. For example neurotransmitters like acetylcholine. Endocrine signalling refers to systemic effects of hormones, for example insulin is produced in the pancreatic islets, secreted to the bloodstream and the target cells are adipocytes, hepatocytes and myocytes (3).

Historically, descriptions of different hormonal effects have been simplified explanations according to the idea that one hormone has one action. This longstanding belief is, however, changing as the knowledge of the growing number of diverse effects exerted by known hormones is increasing. For example, it has recently been shown that insulin also has other target cells in the brain and the effects extend far wider than glucose homeostasis and may even influence cognitive function, for a review see (4).

1.2 Melatonin, general aspects

Melatonin is an indoleamine derived from tryptophan (Figure 1), two enzymatic steps from serotonin, most commonly known as the pineal gland neuroendocrine hormone that regulates sleep and circadian rhythm. It was first isolated from bovine pineal gland in 1958 and identified as the factor that can lighten the skin of frogs (5).

In the brain melatonin synthesis follows a circadian rhythm. Through a polysynaptic pathway, signals from the suprachiasmatic nucleus in the anterior hypothalamus, result in the release of noradrenaline, activating adrenoreceptors in the pinealocytes. This increases the activity of the rate-limiting enzyme, arylalkylamine N-acetyltransferase (AANAT), needed for melatonin synthesis (1). Generally, levels of melatonin in plasma rise after sunset, peak at around 02.00 a.m. and then gradually decline to markedly lower levels by morning. Exposure to bright light during the night inhibits melatonin release and results in lower plasma levels (6). During the daytime, melatonin release from the pineal gland is inhibited and levels in plasma are low.
Figure 1. Synthesis of melatonin from tryptophan (left) and kynurenine pathway (right). Enzymatic reactions catalysed by tryptophan hydroxylase (TPH1, TPH2), amino acid decarboxylase (AAD), arylalkylamine N-acetyltransferase (ANAAT) and 5-hydroxyindole O-methyltransferase (HIOMT). Enzymatic reactions driving the kynurenine pathway are Indoleamine dioxygenase (IDO), tryptophan-2,3-dioxygenase (TDO) and formamidase.

However, melatonin is also present in peripheral tissue and produced in various organs. The two key enzymes needed for synthesis of melatonin from serotonin, AANAT and hydroxyindole-O-methyl transferase (HIOMT), sometimes referred to as N-Acetylserotonin O-methyltransferase (ASMT), have been found in numerous tissues including retina, stomach, gut, spleen, liver, heart, skeletal muscle, bone marrow, testes, ovaries, placenta, skin and immune cells (7, 8). Most studies however, are conducted in animals and human studies are scarce.

Due to its size and solubility, melatonin can pass easily through cell membranes, including the blood-brain barrier. Therefore, it is not surprising that melatonin is present in essentially all biological fluids: cerebrospinal fluid, saliva, bile, amniotic fluid, synovial fluid and breast milk (7).

The liver and the CYP P450 enzymes constitute the major metabolic pathway for degradation of melatonin (9). The metabolite 6-hydroxymelatonin is conjugated with sulphate or glucuronide before it is secreted in the urine (10). When administered intravenously, melatonin is rapidly distributed and eliminated and the half-life is only 0.5 to 5.6 minutes (11).

1.3 Melatonin receptors, MT<sub>1</sub> and MT<sub>2</sub>

Melatonin has two known receptors, MT<sub>1</sub> and MT<sub>2</sub>. Both are membrane bound G-protein coupled receptors and act by affecting second messengers (12). A schematic image of a G-protein coupled receptor is shown in Figure 2. The receptors are widely distributed and have been identified in countless tissues and organs, mostly in animals, see review (13-15). A summary of the
current knowledge of the distribution of melatonin receptors in normal human tissues is presented in Table 1.

Melatonin can also interact with nuclear receptors belonging to the retinoid related orphan nuclear hormone receptor (ROR/RZR) subfamily (16). Melatonin’s abilities to influence transcription may be at least in part dependent on the interaction with these nuclear receptors (17).

**Figure 2.** Schematic image of a G-protein coupled receptor like MT₁ and MT₂. When a hormone binds to the receptor, the G-protein is activated, transferring the extracellular signal to the inside of the cell where an enzyme cascade is activated. The G-protein consists of three subunits, α, β and γ. In the resting state, these subunits form a complex. When the receptor is activated, the α-subunit dissociates, activating one or more target enzymes. The dissociation is catalysed by GTPase, which converts GTP to GDP, releasing energy.

GTP = guanosine triphosphate, GDP = guanosine diphosphate, cAMP = cyclic adenosine monophosphate.
Table 1. Summary of distribution of melatonin receptors, $MT_1$ and $MT_2$ in normal human tissue and putative effects.

<table>
<thead>
<tr>
<th>Organ/tissue</th>
<th>Receptor</th>
<th>Method</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain (SCN, hippocampus, cerebellum)</td>
<td>$MT_1$, $MT_2$</td>
<td>PCR, IHC</td>
<td>Regulate neuronal activity and circadian rhythm</td>
<td>(18-21)</td>
</tr>
<tr>
<td>Retina</td>
<td>$MT_1$, $MT_2$</td>
<td>PCR, IHC (MT$_1$)</td>
<td>Regulate dopaminergic and GABAergic transmission</td>
<td>(22)</td>
</tr>
<tr>
<td>Immune system (lymphocytes)</td>
<td>$MT_1$</td>
<td>PCR</td>
<td>Promote immune cell proliferation, stimulate IL2 production</td>
<td>(23)</td>
</tr>
<tr>
<td>Brown adipose tissue</td>
<td>$MT_1$, $MT_2$</td>
<td>PCR</td>
<td>Regulate adipocyte physiology, decrease Glut4</td>
<td>(24)</td>
</tr>
<tr>
<td>Salivary glands</td>
<td>$MT_1$</td>
<td>IHC</td>
<td></td>
<td>(25)</td>
</tr>
<tr>
<td>Cardiovascular system</td>
<td>$MT_1$, $MT_2$</td>
<td>PCR</td>
<td>Vasoconstriction ($MT_1$) Vasoconstriction ($MT_2$)</td>
<td>(26-28)</td>
</tr>
<tr>
<td>Breast</td>
<td>$MT_1$</td>
<td>IHC</td>
<td></td>
<td>(19)</td>
</tr>
<tr>
<td>Kidney</td>
<td>$MT_1$, $MT_2$</td>
<td>PCR</td>
<td>Regulation of glomerular filtration</td>
<td>(29)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>$MT_1$ (α-cells) $MT_2$ (β-cells)</td>
<td>PCR</td>
<td>Regulate glucose homeostasis</td>
<td>(30, 31)</td>
</tr>
<tr>
<td>GI tract</td>
<td>2[125I]iodomelatonin binding sites $MT_1$ (colon)</td>
<td>Autoradiography, PCR, IHC, western blot</td>
<td>Motility, paracellular permeability, secretion of HCO$_3^-$</td>
<td>(32, 33)</td>
</tr>
<tr>
<td>Gallbladder</td>
<td>$MT_1$</td>
<td>PCR, western blot, IF</td>
<td>Protect against oxidative stress</td>
<td>(34)</td>
</tr>
<tr>
<td>Ovary</td>
<td>$MT_1$, $MT_2$</td>
<td>PCR</td>
<td>Regulate ovarian and reproductive function</td>
<td>(35)</td>
</tr>
<tr>
<td>Uterus</td>
<td>$MT_1$, $MT_2$</td>
<td>PCR, in situ hybridization</td>
<td>Regulate myometrical contractility</td>
<td>(36)</td>
</tr>
<tr>
<td>Prostate</td>
<td>Functional binding sites</td>
<td>Autoradiography</td>
<td></td>
<td>(37)</td>
</tr>
<tr>
<td>Skin</td>
<td>$MT_1$, $MT_2$</td>
<td>PCR</td>
<td>Protect against UV-induced damage</td>
<td>(38)</td>
</tr>
</tbody>
</table>

Modified from (39).
1.4 Melatonin and the brain

Melatonin is well known to regulate sleep and circadian rhythm and in psychiatry, both melatonin and agomelatin (a melatonin receptor agonist) are frequently used to treat mood disorders and sleep disturbances. As shown in Figure 3, aging is associated with disrupted sleep and both circadian rhythm and nocturnal melatonin amplitude declines with age (40).

![Figure 3. Melatonin levels in daytime and nighttime during aging (41)](image)

Alterations of the normal circadian secretory pattern of melatonin have been demonstrated in several psychiatric disorders. It is, however, unclear whether they represent a cause or are a consequence of these disorders (42).

Melatonin may also be involved in cognitive functions and middle-aged patients with cognitive impairment displayed lower median nocturnal melatonin response than healthy controls and a tendency to phase advancement (43). Deranged circadian rhythms as well as lower melatonin levels have also been found in other neurodegenerative conditions such as Alzheimer’s disease and Parkinson’s disease (44, 45). In patients with severe depression, evening melatonin levels in saliva are reduced (46) and reduced levels of melatonin in patients with affective disorder also appear to be related to functional features such as verbal memory (47). Interestingly in all these conditions, neuroinflammation has been proposed as a contributor to the pathogenesis (48).

Degradation of tryptophan along the kynurenine pathway results in kynurenine and several metabolites that modulate inflammation and have been linked to depression and suicidal behaviour (49). Proinflammatory cytokines lead to upregulation of enzymes (IDO and TDO) that increase the levels of kynurenine and the metabolite, quinolinic acid (QUIN), which has been associated with disorders such as Alzheimer’s disease, multiple sclerosis and psychiatric disorders (50). Another metabolite, kynurenic acid (KYNA) is unable to cross the blood brain barrier and thus constitutes a degradation route that protects the brain from the stress-induced damage of kynurenine. By physical exercise, the enzyme converting kynurenine to KYNA is upregulated, which can explain the mechanisms behind the positive effects of physical exercise on depression (51).

1.5 Melatonin in the gastrointestinal tract

In the gastrointestinal tract production of melatonin has been suggested in the enterochromaffin (EC) cells (52). The EC cells are neuroendocrine cells that also produce serotonin and they func-
tion as sensory cells in the gastrointestinal tract, “tasting” the luminal contents and responding by releasing various substances (53). Gastrointestinal melatonin levels have been estimated to exceed that in the pineal gland by 400 times and does not seem to be controlled by the circadian clock but rather related to fasting and food intake (54). For example postprandial elevations of melatonin in human saliva have been detected (55). Pinealectomy did not reduce levels of gastrointestinal melatonin, indicating that gut melatonin is independent from pineal production (56). Administration of exogenous melatonin, or its precursors increase the amount of gastrointestinal melatonin (57).

Little is known about the localisation and distribution of melatonin receptors in human gastrointestinal tract. One study on human duodenum found that melatonin increased intracellular calcium (Ca$^{2+}$), possibly through the MT$_2$ receptor subtype, as the effect was abrogated by an MT$_2$-selective receptor antagonist (58). Another study has identified MT$^1$ immunoreactivity (IR) in human colon (33). In rats, mRNA transcripts of MT$_1$ were found to be highest in the epithelial and subepithelial layers of the duodenum (59). Levels of MT$_2$ protein expression, however, were found to be highest in colonic smooth muscle (60). Actions of melatonin in the gastrointestinal tract include regulation of intestinal motility, paracellular permeability protection of gastrointestinal mucosa and glucose homeostasis. Various substances are involved in the regulation of gastrointestinal motility. Melatonin and serotonin cooperate through a feedback system in maintaining balance where melatonin appears to act as a physiological antagonist of serotonin, mainly dampening motility (61, 62).

Significant protective effects against stress-induced damage to the intestinal mucosa have been attributed to melatonin. Luminal melatonin for example reduces mucosal paracellular permeability in the duodenum (63), preventing transepithelial leakage of inflammatory substances. Furthermore, binding of luminal melatonin to the MT$_2$ receptor increase bicarbonate secretion in the duodenum in response to acidic luminal contents, maintaining the mucosal barrier (64). A novel study in rats demonstrated that administration of melatonin reduced the changes in small intestinal microvasculature induced by systemic inflammation, probably by limiting the recruitment of local immune cells (65). The authors proposed a therapeutic benefit of melatonin in mild systemic inflammation.

1.6 Other regulatory functions

1.6.1 Immunomodulatory functions

Melatonin has powerful anti-inflammatory properties and acts as a scavenger of free radicals, neutralising reactive oxygen species (66). The anti-oxidative effects of melatonin have been described as 1) a direct free radical scavenger activity, with a cascade of melatonin metabolites, were several of them act as direct free radical scavengers and 2) an indirect anti-oxidative effect due to increased gene transcription, expression and activity of antioxidant enzymes. Through binding of melatonin to membrane bound receptors, cAMP response element binding protein is inhibited and thus transcription of antioxidant genes is regulated.

Several immune cells contain or produce melatonin, for example human lymphocytes produce large amounts of melatonin (67). In the immune system, melatonin acts as a modulator. A recent review indicates that melatonin can act stimulatory on the immune system in immunosuppressive states and exercise anti-inflammatory effects in conditions with acute inflammation (68). Melatonin and serotonin are both ubiquitous in the gut and both are highly involved in modulation of the immune system. Serotonin has seven known receptors, where four have been identified in the gastrointestinal tract (5-HT$^2_R2$, 5-HT$^2_R3$, 5-HT$^2_R4$ and 5-HT$^2_R7$). The immunomodulatory properties of serotonin appear to be mainly stimulatory and administration of serotonin receptor antagonists can attenuate intestinal inflammation (69).
1.6.2 Glucose homeostasis
Melatonin appears to influence glucose homeostasis, for example by affecting transcription factors involved in insulin secretion (70) and mutations in the gene encoding the MT<sub>2</sub> receptor, resulting in impaired melatonin signalling increase the risk of type 2 diabetes (31). Differences in the expression of MT<sub>1</sub> and MT<sub>2</sub> in pancreatic cells have been found. MT<sub>1</sub> mRNA has been identified mainly in the glucagon secreting cells of the pancreas, whereas MT<sub>2</sub> mRNA seems to be primarily located in the insulin producing cells (30, 31).

1.7 Melatonin and cancer
The risk of developing cancer is higher in shift workers exposed to light at night, which is thought to be at least partly due to the suppression of nocturnal production of melatonin in the pineal gland (71). Melatonin exhibits strong oncostatic effects in various types of cancer, including anti-proliferative (72, 73) and pro-apoptotic actions (74). In normal tissue however, melatonin has the opposite effect, promoting survival and inhibiting apoptosis (75).

Most of the previous studies of melatonin and cancer have been conducted in breast cancer and related to the regulation of different hormone receptors. However, a growing body of evidence points to melatonin’s ability to inhibit cancer growth by influencing several different signalling pathways. For example by up regulation of the cell cycle regulatory protein p27kip1 in prostate cancer, which could inhibit tumour proliferation (76). In haematological malignancies melatonin appears to induce apoptosis by activation of the intrinsic as well as the extrinsic pathway (77). In human MCF-7 breast cancer cells melatonin decrease cell proliferation by increase of the expression of p53 and p21WAF1 proteins, inducing cell cycle arrest (78).

1.8 Small intestinal neuroendocrine tumours
EC cells are hormone-producing cells located throughout the gastrointestinal tract. When these cells become malignant they form neuroendocrine tumours, which also produce hormones. Small intestinal neuroendocrine tumours (SI-NET) are characterised by their low proliferation rate and long survival expectancy, yet high propensity to metastasise to the mesentery and to the liver. The tumours are rare, the incidence is 1.12 per 100 000 inhabitants in Sweden (79).

SI-NETs produce and secrete serotonin and other bioactive agents such as tachykinins (80), causing the “carcinoid syndrome”, a set of symptoms including diarrhoea, cutaneous flush, carcinoid heart disease and bronchial constriction. At the time of diagnosis, metastases have generally already formed.

Diagnosis of SI-NETs is based on the analysis of Chromogranin A (CgA) and urinary 5-hydroxyindoleacetic acid (U-5-HIAA), radiology and histopathological analysis (81). Surgery is the primary treatment for SI-NETs and can delay recurrence of the disease and reduce symptoms. When the tumour has spread, surgery is still of great benefit to prevent complications of fibrosis, such as intestinal obstruction and bowel ischemia (82). Medical treatment includes somatostatin analogues and/or interferon-alpha and aims to ameliorate symptoms caused by hormone production and delay progression of tumour growth (83-85).

Interestingly, although melatonin is only two enzymatic reactions from serotonin, the production of melatonin in these tumours is yet to be investigated.
2. Aims of the studies

Based on previous studies in animals, we hypothesised that melatonin is produced in human enterochromaffin cells and consequently that small intestinal neuroendocrine tumours, derived from enterochromaffin cells, also produce melatonin.

The specific aims were:

1. to characterise the tissue expression of melatonin and its receptors in normal human gastrointestinal tract and pancreas
2. to characterise the expression of melatonin and its receptors in small intestinal neuroendocrine tumours
3. to investigate if there were any correlations between levels of circulating melatonin, symptoms, prognostic factors and treatment response.
3. Materials and methods

3.1 Patient samples

3.1.1 Study I
Human tissue, representing different parts of the gastrointestinal tract, was obtained from resection margins removed from adult patients during surgery for malignancies. Only tissue with normal macro- and microscopic appearance was included in the study. Tissue biopsies from the gastrointestinal tract of 39 individuals and from the pancreas of 3 individuals were used (17 purchased from Asterand, Detroit, MI, USA and 25 from the Department of Pathology, Uppsala University Hospital, Uppsala, Sweden). Tissue sections from the gastrointestinal tract represented stomach (n=12), small intestine (n=11), appendix (n=3) and large intestine (n=13). The identity and clinical data of the patients were not known and was not included in the analyses.

3.1.2 Study II
Tumour tissue from patients with SI-NETs, consisting of serial sections from primary tumours and metastases was obtained from patients diagnosed at the Laboratory of Pathology and Cytology and treated at the Department of Endocrine Oncology, Uppsala University Hospital in Sweden. Only patients where paired sections of primary tumour and metastasis could be obtained were included in the analyses (n=26).

Plasma samples from patients with SI-NETs were collected at two time points, before and after treatment. All samples were collected in the morning, from fasting patients. Only patients where sampling results were available for both sampling occasions were included in statistical analyses (n=43), see flow chart in Figure 3.

Clinical data was collected for all patients with SI-NETs included in histological or plasma analyses. Patient records were systematically examined and data was extracted regarding age at diagnosis, body mass index, smoking history, diagnosis of diabetes, use of psychiatric drugs, Ki67 and levels of U-5-HIAA and CgA at the time of operation or plasma sampling.

![Figure 3. Visualisation of included patients. Clinical records were collected for 85 patients with SI-NETs, plasma samples were available for 46 patients and tumour tissue was available for 39 patients. Only individuals with plasma samples from both sampling occasions were included in plasma analyses (n=43). Only patients where tumour tissue was available from primary tumour as well as metastasis were included in tissue analyses (n=26). Seventeen patients were included in both analyses. SI-NETs = small intestinal neuroendocrine tumours](image-url)
3.2 Immunohistochemistry

For detailed information regarding the immunohistochemistry methods, see respective publication (86, 87), here a summary follows. Tissue specimens were fixed in 4% buffered formalin, dehydrated, and embedded in paraffin wax. Sections, 4 µm thick, were attached to positively charged glass superfrost slides (Menzel-Gläser, Braunschweig, Germany). Sections were deparaffinised in xylene and rehydrated to distilled water. Antigen retrieval was performed using pressure cooker treatment for 10 minutes in citrate buffer pH 6.

Sections were incubated with antibodies for melatonin, MT₁, MT₂ and serotonin, for details see Table 2. Diaminobenzidine was used as chromogen. For visualisation of nuclei, the sections were counterstained with Mayer’s haematoxylin, for details see (86).

For double immunofluorescence staining, sections were prepared as described above and then incubated overnight at 4°C with a cocktail of two primary antibodies (dilutions were as follows: anti-MTR-1A at 1:50, anti-melatonin receptor 1B at 1:50, anti-serotonin 1:50, anti-melatonin at 1:100). Before application of the antibody cocktail, the sections were incubated with a non-immune serum from the animal species producing the secondary antibody, diluted 1:10 in PBS with 1% BSA. The secondary antibodies used were: tetramethyl rhodamine isothiocyanate (TRITC)-conjugated rabbit anti-goat TRITC (Alexa 555 A 21431) fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse (Alexa 488 A 11001) TRITC-conjugated goat anti-rabbit (Alexa 555 A 21428) and FITC-conjugated rabbit anti-mouse (Alexa 488 A 11059) all from Life Technologies Europe BV (Stockholm, Sweden). The incubation time for the secondary antisera was 30 minutes at room temperature. The sections were examined in a Zeiss 510 confocal microscope and photographed with an AxioCam camera, employing ZEN 2012 imaging software (Carl Zeiss AB, Stockholm, Sweden) and a 40X plan-apochromat objective. Co-localisation studies were performed on tissue sections from the pylorus (n = 2) and ileum (n = 2).

<table>
<thead>
<tr>
<th>Target</th>
<th>Antibody</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melatonin</td>
<td>0100–0203</td>
<td>1:500</td>
<td>AbD Serotec, Kidlington, UK</td>
</tr>
<tr>
<td>MT₁</td>
<td>Anti-MTR-1A, (N-20), sc13179</td>
<td>1:500</td>
<td>Santa Cruz Biotechnology, Dallas, Texas, USA</td>
</tr>
<tr>
<td>MT₂</td>
<td>ABIN122307</td>
<td>1:100</td>
<td>Antibodies-online GmbH, Aachen, Germany</td>
</tr>
<tr>
<td>Serotonin</td>
<td>Clone 5HT-H209</td>
<td>1:100</td>
<td>DAKO Sweden AB, Stockholm, Sweden</td>
</tr>
</tbody>
</table>

3.2.1 Study I. Histological assessment

All specimens were coded and examined microscopically by two independent observers. IR intensity was classified as negative, weak, medium or strong for melatonin, MT₁ and MT₂ and the cellular and intracellular localisation was documented. Sections from skin were used as a positive control for MT₁ and MT₂.

3.2.2 Study II. Histological assessment

Sections were coded and examined microscopically by three different observers, performed blinded at different occasions. Intensity of IR was classified as negative, weak, moderate and strong. Intraclass correlation coefficient was 0.955 between evaluations of different observers. The sections were also analysed using Image J software to further ensure reliability in scoring. When comparing manual scoring with the computerised scoring, intraclass correlation coefficient was 0.934.

3.3 Microarray analysis

Raw data from the public gene expression data archive Gene Expression Omnibus (GEO) (88) was extracted from small intestinal epithelium GEO series GSE9576 (89) and from human pan-
creas from the GEO series GSE16515 (90) and GSE15471 (91). From a different platform, normal pancreatic islet samples GEO Series GSE3842 (92) were obtained and the samples were imported into Expression Console, see Table 3 for a summary of the series. Normalisation was performed using the robust multi-array average method (93). One sample failed the quality controls and was excluded. Normalized Log2 expression signals were then extracted.

**Table 3. Summary of gene expression series from the Gene Expression Omnibus data archive used in the microarray analyses.**

<table>
<thead>
<tr>
<th>GEO series</th>
<th>Tissue</th>
<th>Platform</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSE9576</td>
<td>Small intestinal epithelium</td>
<td>Affymetrix Human Genome U133 Plus 2.0 Array (GEO Accession GPL570)</td>
<td>(89)</td>
</tr>
<tr>
<td>GSE16515</td>
<td>Pancreas</td>
<td>Affymetrix Human Genome U133 Plus 2.0 Array (GEO Accession GPL570)</td>
<td>(90)</td>
</tr>
<tr>
<td>GSE15471</td>
<td>Pancreas</td>
<td>Affymetrix Human Genome U133 Plus 2.0 Array (GEO Accession GPL570)</td>
<td>(91)</td>
</tr>
<tr>
<td>GSE3842</td>
<td>Pancreas</td>
<td>Affymetrix Human Gene 1.0 ST Array (GEO Accession GPL6244)</td>
<td>(92)</td>
</tr>
<tr>
<td>GSE11223</td>
<td>Colon</td>
<td>Agilent-012391 Whole Human Genome Oligo Microarray G4112A</td>
<td>(94)</td>
</tr>
</tbody>
</table>

Gene expression analyses were performed using the statistical computing language R and data for enzymes involved in melatonin synthesis (TPH1-2, TDO2, IDO1, DDC, AANAT, HIOMT (ASMT), receptors for melatonin (MTNR1A, MTNR1B) and serotonin (5-HTR1-7) were extracted. Expression signals for parathyroid hormone (PTH) were used as a negative control, see Table 4 for summary of genes analysed.

Differences in gene expression between colon biopsies from patients with ulcerative colitis (inflamed) and healthy controls (not inflamed) from the GEO series GSE11223 (94) was investigated using The Linear Models for Microarray Analysis (limma) R package on GEO2R.

**Table 4. Summary of genes analysed using microarray analysis.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Main function</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPH1</td>
<td>Tryptophan hydroxylase 1</td>
<td>Converts Trp to 5-OH-Trp</td>
</tr>
<tr>
<td>TPH2</td>
<td>Tryptophan hydroxylase 2</td>
<td>Converts Trp to 5-OH-Trp</td>
</tr>
<tr>
<td>TDO2</td>
<td>Tryptophan-2,3-dioxygenase</td>
<td>Converts Trp to N’-formyl kynurenine</td>
</tr>
<tr>
<td>IDO1</td>
<td>Indoleamine dioxygenase</td>
<td>Converts Trp to N’-formyl kynurenine</td>
</tr>
<tr>
<td>DDC</td>
<td>Amino acid decarboxylase</td>
<td>Converts 5-OH-Trp to serotonin</td>
</tr>
<tr>
<td>AANAT</td>
<td>Arylalkylamine N-acetyltransferase</td>
<td>Converts serotonin to N-acetyl serotonin</td>
</tr>
<tr>
<td>HIOMT</td>
<td>5-hydroxyindole O-methyltransferase</td>
<td>Converts N-acetyl serotonin to melatonin</td>
</tr>
<tr>
<td>MTNR1A</td>
<td>Melatonin receptor type 1</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>MTNR1B</td>
<td>Melatonin receptor type 2</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>5-HTR1</td>
<td>5-HT receptor 1</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>5-HTR2</td>
<td>5-HT receptor 2</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>5-HTR3</td>
<td>5-HT receptor 3</td>
<td>Ligand-gated ion channel</td>
</tr>
<tr>
<td>5-HTR4</td>
<td>5-HT receptor 4</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>5-HTR5</td>
<td>5-HT receptor 5</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>5-HTR6</td>
<td>5-HT receptor 6</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>5-HTR7</td>
<td>5-HT receptor 7</td>
<td>G-protein coupled receptor</td>
</tr>
</tbody>
</table>

3.4 Hormone analyses

In study II, plasma samples were collected after an overnight fast at two time points and stored at -80°C. Measurements were performed using competitive radioimmunoassay (Melatonin direct RIA, LDN, Norhorn, Germany).

Medical and surgical treatment between samplings was documented. Response to treatment was classified as regression, stabilisation or progression, according to radiological and/or bio-
chemical response. In statistical analyses, patients with stabilisation or tumour regression were compared to those with tumour progression.

3.5 Statistical analyses

In study II, data was analysed using the statistical program package SPSS 21.0 (SPSS Inc., Chicago, IL, USA). Statistical analyses were conducted separately for the two cohorts; those where tumour tissue specimens were available (n = 26) and those where plasma samples were collected (n = 43). The two cohorts were not compared to each other.

Survival was calculated from the time of diagnosis to death. Survival analyses were performed using the Mantel-Cox log rank test. For comparison of intensity in primary tumours and metastases and for change in biomarker response between plasma samplings, Wilcoxon Signed Ranks Test was used. For correlations between IR or plasma levels of melatonin and clinical parameters Spearman’s test was used. The Mann-Whitney U test was used to look for sex differences in circulating plasma levels of melatonin.
4. Results and discussion

4.1 Expression of melatonin and its receptors in normal human gastroenteropancreatic tract and in SI-NETs

Positive IR for melatonin was seen in EC cells throughout the gastrointestinal tract. This is in agreement with previous findings in animals, where the enzymes needed for melatonin synthesis have been identified in EC cells (52).

In SI-NETs, derived from EC cells, melatonin IR was seen in all sections representing primary tumour and metastases. The intensity however, varied from weak to strong. In support of the immunohistochemical results, gene expression for the enzymes involved in melatonin synthesis, AANAT and HIOMT, was found in epithelium from human small intestine enriched for EC cells.

In plasma from patients with SI-NETs, the median level of melatonin was 26.0 pg/L [4.5–220.0] at the first sampling and 23.0 pg/L [8.9–90.7] at the second sampling. It has been proposed that the pineal gland is responsible for nocturnal circulating levels of melatonin. However, in the daytime, levels of melatonin in plasma appears to be gut derived (57). With the hypothesis that SI-NETs express and produce melatonin, higher levels of plasma melatonin were to be expected. In patients with disease regression or stabilisation, circulating levels of melatonin were reduced at the second sampling (r = 0.356; p = 0.038). This may be due to a reduction in tumour burden resulting in less tumour-derived melatonin.

No expression of MT1 IR was seen in EC cells. MT2 IR however, was present in EC cells in some of the gastric sections and in all sections from small and large intestine. This finding was confirmed in the gene expression data where the MT2 mRNA levels were much higher than MT1 mRNA levels.

In tumour cells, MT1 IR was also weak or absent. Positive IR for MT2 was found in all sections from primary tumour and all but two from metastasis. The IR intensity of MT2 was generally lower in metastases than in primary tumours (r=0.53; p=0.007).

Melatonin is in contrast to serotonin not stored in secretory vessels and easily passes over membranes. In light of this, it was not surprising that we found diffuse epithelial melatonin IR with varying intensity throughout the gastrointestinal tract. There appeared, however, to be a gradient in the GI-tract as most sections representing stomach and small intestine displayed none or weak IR, while sections from the large intestine generally displayed high intensity IR in the cytoplasm of epithelial cells as well as in the mucus layer lining the epithelium. In addition to EC cell derived melatonin, many foods, both vegetable and animal contain melatonin (95), why some of this mucosal melatonin may originate from digested foods, accumulating in the gastrointestinal lumen (96). Also, microorganisms produce melatonin (97) and considering that the gastrointestinal tract harbours about $10^{14}$ indigenous microbes (98) this probably further contributes to the luminal contents of melatonin. In the current study, the definitive origin of the mucosal melatonin IR cannot be determined.

Melatonin exhibits many important functions in protecting the gastrointestinal mucosa and maintaining the epithelial barrier. In addition to reducing paracellular permeability and increasing bicarbonate secretion, which have been demonstrated in the duodenum, a more general protective effect is probably mediated via melatonin’s free radical scavenging properties, protecting lipids, proteins and nuclear DNA from oxidative damage (99). It has also recently been demonstrated that melatonin diminishes the changes to the microvasculature caused by systemic inflammation, especially in the small intestine (65). Melatonin may also be involved in visceral
sensation mediation and experimental studies have indicated a beneficial effect of melatonin administration on abdominal pain, rectal pain sensitivity, as well as extra colonic symptoms in patients with irritable bowel syndrome (100, 101).

Interestingly, in SI-NETs, high melatonin IR intensity, both in primary tumours and metastases, correlated to less reported symptoms of diarrhoea \( (r=-0.484; \ p=0.012 \text{ and } r=-0.398; \ p=0.044, \text{ respectively}) \). SI-NETs are known to cause disabling diarrhoea, with up to 10 watery stools daily. Our findings are congruent with the known actions of melatonin, as its effects on gastrointestinal motility are predominantly inhibitory, and melatonin can slow down colonic transit (102). Higher levels of melatonin in plasma also correlated to the symptoms of nausea and/or vomiting at both sampling occasions \( (r=0.337; \ p=0.027 \text{ and } r=0.413; \ p=0.006, \text{ respectively}) \) and to flush at the second sampling \( (r=0.353; \ p=0.020) \). It can be speculated that a slower intestinal transit would theoretically cause nausea. There are other potential explanations for the symptom of nausea, for example serotonin can induce nausea. However, there was no correlation between the levels of U-5-HIAA at the time of sampling and nausea. At the second sampling, plasma levels of melatonin correlated to the symptom of flush. Although other active substances, such as tachykinins are involved in the development of flush (80), melatonin can also exert vasodilator effects by binding to the MT\(_2\) receptor in arterial smooth muscle (28).

In the epithelium, IR for both MT\(_1\) and MT\(_2\) was strong in the large intestine, in the cytoplasm as well as in the nucleus. In other cell types, MT\(_2\) IR was generally higher than MT\(_1\) IR. Previously, studies of gastrointestinal receptor expression have mainly been conducted in animals. In rats MT\(_1\) mRNA was reported primarily in the sub epithelial layers of the gut (muscularis externa and serosa) and the mRNA levels in epithelial tissue were generally low (59). Considering that gastrointestinal melatonin levels vary with fasting and food intake there is a possibility that different behaviour regarding feeding in animals versus humans may affect the receptor expression through a feedback mechanism. The signal transduction pathways involved in melatonin binding to its receptors are complex and vary within cells and possibly also among species, which could explain differences in receptor expression.

Melatonin exerts important anti-inflammatory actions and in critically ill children, elevated plasma levels have been speculated to represent a physiologic response to the oxidative stress of serious diseases (103). In this context, up-regulation of melatonin receptors or the enzymes needed for melatonin synthesis would have been expected in inflamed tissue from patients with inflammatory bowel disorder. However, no significant difference was seen between ulcerative colitis and controls in the microarray analysis. As an indication of the increased inflammation, higher levels of the genes encoding the enzymes converting tryptophan to kynurenine was noted in the inflamed tissue compared to controls.

4.2 Melatonin and its receptors in human pancreas

Strong melatonin IR was additionally found in endocrine cells of the pancreatic islets. The expression of MT\(_1\) IR varied, one section showed strong IR in a subset of cells while the other two sections were negative. Positive MT\(_2\) IR was also seen in a subset of cells in the endocrine pancreas. Expression of the genes encoding MT\(_2\) was higher than those of MT\(_1\) in pancreas, which is in line with the findings in the immunohistochemistry analyses.

Recent studies have demonstrated that melatonin signalling in pancreatic islets appears to be of importance for the regulation of survival and function of \( \beta \)-cells and the effect seems to be mediated by the MT\(_2\) receptor. Our findings support earlier studies of MT\(_2\) receptor expression in human pancreatic islets (30). Activation of melatonin receptors in the \( \beta \)-cells could provide a therapeutic target for patients with type 2 diabetes.
4.3 Melatonin and its receptors in patients with SI-NETs, anti-tumourigenic effects

Melatonin IR was seen in all sections representing primary tumours and metastases. The intensity varied from weak to strong. An inverse correlation between melatonin IR intensity and Ki67 ($r=-0.446; p=0.022$) was found, which is in agreement with the described oncostatic effects of melatonin. Several studies have investigated the anti-tumourigenic effects of melatonin and it has been proposed that these effects include suppression of proliferation, induction of apoptosis, anti-metastatic actions and a drive towards cell differentiation. In prostate cancer cells, administration of melatonin reduced cell growth, stopped cell cycle progression and induced cellular differentiation and the effects appeared to be receptor-independent (104).

In our study receptor IR in tumour tissues varied, with weak or absent MT$_1$ IR while MT$_2$ IR was found in almost all tumour sections, although the intensity was lower in metastases. Studies investigating melatonin receptor expression in other types of cancer have found varying results. For example in ovarian cancer, MT$_1$ was expressed but the expression did not correlate to survival or other outcome measures, which indicates a limited prognostic significance (105). In breast cancer and prostate cancer cells on the other hand, the growth inhibitory effects of melatonin have in some studies been linked to the MT$_1$ receptor (76, 106). Based on these previous studies, higher expression of MT$_1$ could have been anticipated. However, the expression of the MT$_2$ receptor in cancer cells is not as extensively investigated and its role in tumourigenesis is still unclear. In our study, MT$_2$ IR was abundant in SI-NETs and generally weaker in metastases. This down regulation of receptors could indicate a reduced sensitivity to the anti-proliferative effects of melatonin with increasing malignant progression.

4.4 Plasma levels of melatonin in patients with SI-NETs and the brain

Given that melatonin is frequently used in psychiatric care, both for depression and for treating sleep disturbances, we expected to find some kind of psychiatric manifestation in patients with higher levels of melatonin. We examined the prevalence of medication for depression, anxiety or sleep disturbances. However, no associations were seen. Unfortunately, the design of the study did not allow for reliable evaluation of more complex psychiatric symptoms, and information concerning mental health was not routinely requested upon visits.
5. Methodological considerations

Immunohistochemistry is a method based on antibodies specific to the hormone or protein that is to be identified. One limitation with immunohistochemical methods is the risk of cross-reactivity, in which the antibody binds to something similar to the intended target. When dealing with small molecules, such as melatonin and serotonin, control experiments are key to increase reliability of the method. We performed antibody neutralisation tests, where the melatonin antibody was preincubated with both melatonin and serotonin. The antibody against melatonin also displayed partial IR for serotonin indicating that there is some level of cross-reactivity. In study I co-localisation studies were conducted to minimize this confounding factor and there was only a partial overlap between IR for melatonin and serotonin. The lack of correlation between IHC results for serotonin and melatonin performed on serial sections also indicates that the antibodies are identifying separate targets. The antibodies against MT$_1$ and MT$_2$ were preincubated with the corresponding peptides.

In study I, tissue specimens were obtained from patients undergoing surgery for different kinds of gastrointestinal malignancies. The age and sex of the patients were not known, which could potentially affect the expression of melatonin or melatonin receptors. The malignancies themselves could also possibly affect the expression, through locally produced compounds for example cytokines and other inflammatory agents that may cause up- or down regulation of melatonin receptors or increased melatonin production.

Pancreatic tissue was only available from three patients and the receptor expression varied, especially for the MT$_1$ receptor. This variation further raises the question of other factors influencing the receptor expression and biopsies from a higher number of individuals would be desirable to draw any firm conclusions.

Consumption of melatonin rich food results in elevated levels of circulating plasma melatonin (107). Plasma samples were collected after an overnight fast, which minimizes the interaction of dietary melatonin and postprandial elevations, but it is still possible that differences in diet could affect the measured levels of plasma melatonin. Microbial flora is another potential source of melatonin.
6. Conclusions

This thesis has identified melatonin and its receptors throughout the normal human gastrointestinal tract and pancreas, as well as in malignant small intestinal neuroendocrine tumours derived from enterochromaffin cells. The receptor expression varied in both normal and malignant tissues. Melatonin immunoreactivity intensity in tumours correlated significantly to less diarrhoea and to lower proliferation index but not to survival.

Plasma levels of melatonin were reduced with treatment response defined as tumour stabilisation or regression, indicating a possible tumour-derived origin of circulating melatonin levels.

These findings are well in line with the suggested actions of melatonin on gastrointestinal motility and tumour growth.
7. Future studies

In this work we identified the expression of melatonin and its receptors in the normal human gastrointestinal tract and in malignant hormone producing tumours. We also found that higher intensity of melatonin IR in tumour tissue not only correlated to lower proliferation index but also to less symptoms of diarrhoea. Higher levels of melatonin in plasma correlated to more trouble with nausea. These findings are in agreement with the known functions of melatonin in the gut, affecting motility and secretion and further raise the question of a link between melatonin and gastrointestinal symptoms. In conditions where melatonin levels are altered, especially during daytime, is gastrointestinal function also altered and how is this manifested?

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder, where the sufferer experience pain or discomfort and variations in stool consistency. The pathophysiology in IBS is not fully understood but so far no reliable biomarkers have been identified. The patients suffer nonetheless and quality of life is often greatly affected. Many studies have reported a high psychiatric comorbidity in patients with IBS, with primarily depression and anxiety. Few studies however, have investigated the link from a psychiatric perspective.

Levels of melatonin at night are reduced in patients with depression and a recent study by Sundberg et. al. from our group of researchers found that lower bedtime melatonin levels correlated to symptom severity in depressed patients (46). A link between immune system dysregulation and depression has earlier been proposed and as melatonin has many immunomodulatory actions, the influence of gastrointestinal melatonin on paracellular permeability, immune system activity and depressive disorders remains elusive.

To further clarify the connection between gastrointestinal symptoms and psychiatric disorders we aim to design a study to 1) investigate the prevalence of gastrointestinal symptoms in a population of young patients with depression and anxiety disorders and 2) to study the correlation between levels of melatonin in saliva during the day and gastrointestinal symptoms. For more information see the attached research plan.
8. Acknowledgements

Firstly, I would like to express my deepest gratitude to my acting supervisor, Janet Cunningham, for taking me on as a medical student and then as a PhD candidate. Thank you for the continuous support, for your patience, encouragement, inspiration and never-ending curiosity. I could not have imagined having a better supervisor and mentor. I would also like to thank my associate supervisor Lisa Ekselius for making this work possible.

Thank you to my co-authors, Eva Tiensuu Janson, Per Hellström, Mats Stridsberg, Abir Ali and Annica Rasmusson for invaluable knowledge and feedback. My sincere thanks also goes to Åsa Forsberg, laboratory technician for excellent technical assistance, Hans Arinell, statistician at the Department of Neuroscience and Psychiatry and Jeremy Adler at the SciLife BioVis Facility, Uppsala, for assistance in performing the Image J analysis.

Last but not the least I would like to thank my family. My father Lennart, for reading all the drafts with great patience. Thank you for your insightful comments and sharp eye for details. My mother Carina, for your wisdom and soothing tea in times of research stress. My brothers, John and Linus for being who you are and for preventing me from ever feeling lonely growing up. And thank you to my life partner and best friend John and our son Olle for all of the laughter, support and love.
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