The Role of Melatonin in Breast Cancer

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To my children, Eleni and Manos.

For giving me all the right reasons to walk the line.

The Role of Melatonin in Breast Cancer

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ABSTRACT

Melatonin is a hormone secreted by the pineal gland, responsible for the regulation of the circadian rhythm. Beyond its primary function, it has been implicated in various physiological processes and research suggests a potential link between melatonin and malignancy and particularly breast cancer. The aim of this thesis was to investigate the role of melatonin in breast cancer.

Paper I examined the association between melatonin intake and survival in postmenopausal women diagnosed with early breast cancer, assessing the possible protective role of melatonin on survival. While melatonin consumption was associated with improved breast cancer-specific survival in univariable analysis, the effect was no longer significant after adjusting for known predictive and prognostic variables.

Paper II investigated the expression of MT1 melatonin receptor in luminal invasive ductal breast carcinoma in postmenopausal women, in relation to prognostic and predictive factors and survival. No significant associations were observed between MT1 receptor expression and either clinicopathological parameters or survival.

Paper III assessed cancer risk in visually impaired individuals and their matched controls. Visual impairment was associated with increased risk for cancer overall, and for specific types including breast. Various confounding factors could explain this association.

Paper IV evaluated the addition of melatonin to systemic oncologic treatment of patients with metastatic breast cancer, in relation to tumor response and

survival. The analysis revealed significantly improved tumor response rates and 1-year survival among patients receiving melatonin in combination with standard therapy.

In conclusion, melatonin was not found to have a significant effect on the survival of patients with early breast cancer. MT1 receptor expression was not correlated with prognostic or predictive factors or survival. Visual impairment was associated with increased cancer risk overall and for specific cancers. The addition of melatonin to standard treatment in patients with stage IV breast cancer showed enhanced tumor response and improved survival.

Keywords: melatonin, breast cancer, survival, MT1, visual impairment, tumor response, stage IV breast cancer

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SAMMANFATTNING PÅ SVENSKA

Melatonin är ett hormon som utsöndras av tallkottkörteln och ansvarar för regleringen av dygnsrytmen. Utöver sin primära funktion har melatonin en roll i flera fysiologiska processer, och forskning har antytt ett möjligt samband mellan melatonin och cancer, särskilt bröstcancer. Syftet med denna avhandling var specifikt att undersöka melatonins roll vid bröstcancer.

Delarbete I undersökte sambandet mellan förskrivning av melatonin och överlevnad hos postmenopausala kvinnor med tidig bröstcancer, med fokus på melatonins eventuellt skyddande effekt på överlevnad. Även om melatonin var associerad med förbättrad bröstcancerspecifik överlevnad så kvarstod inte effekten efter justering för kända prediktiva och prognostiska variabler.

Delarbete II analyserade uttrycket av melatoninreceptorn MT1 i luminal invasiv duktal bröstcancer hos postmenopausala kvinnor i relation till prognostiska och prediktiva faktorer samt överlevnad. Inga signifikanta samband observerades mellan MT1-receptoruttryck och vare sig klinikopatologiska parametrar eller överlevnad.

Delarbete III utvärderade cancerrisk hos synskadade individer och deras matchade kontroller. Synnedsättning var associerad med en ökad risk för cancer generellt, samt för vissa specifika cancerformer, inklusive bröstcancer. Olika förväxlingsfaktorer kan förklara detta samband.

Delarbete IV utvärderade tillägg av melatonin till systemisk onkologisk behandling hos patienter med metastaserad bröstcancer i relation till tumörrespons och överlevnad. Analysen visade signifikant förbättrad tumörrespons och 1-årsöverlevnad hos patienter som erhöll melatonin i kombination med standardbehandling.

Sammanfattningsvis visades ingen signifikant effekt av melatonin på överlevnaden hos patienter med tidig bröstcancer. MT1-receptoruttryck korrelerade inte med prognostiska eller prediktiva faktorer eller överlevnad. Synnedsättning var associerad med en ökad cancerrisk generellt och för vissa specifika cancerformer. Tillägg av melatonin till standardbehandling hos patienter med stadium IV bröstcancer visade förbättrad tumörrespons och förbättrad överlevnad.

1 LIST OF PAPERS

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

I. Pistiolis L, Khaki D., Kovács A., Olofsson Bagge R.

The Effect of Melatonin Intake on Survival of Patients with Breast Cancer – A Population-Based Registry Study.

Cancers (Basel). 2022;14(23):5884

II. Pistiolis L., Alawieh S., Halldorsdottir T., Kovács A., Olofsson Bagge R.

Melatonin MT1 Receptor Expression in Luminal Invasive Ductal Breast Carcinoma in Postmenopausal Women.

Biomolecules. 2025;15(4):581

III. Pistiolis L., Litsne, H., Olofsson Bagge R., Axelsson, K.F

Visual Impairment and Cancer Risk: A Nationwide Cohort Study of Adult Swedish Men and Women.

Cancers (Basel). 2025;18(1):147

IV. Pistiolis L., Axelsson K.F., Katsarelias D., Olofsson Bagge R.

Effect of Melatonin Addition to Systemic Treatment on Tumor Response and Survival in Patients with Stage IV Breast Cancer: A Systematic Review and Meta-Analysis.

In manuscript form

CONTENTS

ABBREVIATIONS

AANAT	Arylalkylamine N-acetyltransferase
ARR	Absolute risk reduction
BCSS	Breast cancer-specific survival
CCI	Charlson comorbidity index
CDKs	Cyclin-depended kinases
CSF	Cerebrospinal fluid
EBM	Evidence-based medicine
ER	Estrogen receptor
ERE	Estrogen response element
HER2	Human epidermal growth factor receptor 2
IHC	Immunohistochemistry
LAN	Light at night
MT1	Melatonin receptor 1
MT2	Melatonin receptor 2
NHG	Nottingham histologic grade
NNT	Number needed to treat
NPI	Nottingham prognostic index
OS	Overall survival
PR	Progesterone receptor

SCN	Suprachiasmatic nucleus
SEEM	Selective estrogen enzyme modulator
SERM	Selective estrogen receptor modulator
SR/MA	Systematic review and meta-analysis
TP53	Tumor protein 53
WI	Weighted index

1 INTRODUCTION

1.1 ORIGIN AND BIOLOGICAL SIGNIFICANCE OF MELATONIN

Melatonin's discovery represents a pivotal milestone in chronobiology and neuroendocrinology. Until then, the pineal gland was regarded as a vestigial organ, with unclear biological function, shrouded in "mystical" properties of questionable scientific significance. In 1954, J. Kitay and M. Altschule published *The pineal gland: A Review of the Physiologic Literature* (1), which included a summary and analysis of the approximately 1,800 papers on the pineal gland and its functions that had been published until that day. After careful examination, they concluded that the pineal gland was possibly implicated in the following: the skin's color response to light fluctuations in lower vertebrates, the control of the function of the gonads, and behavior. This laid the groundwork for what was to follow.

1.1.1 HISTORICAL PERSPECTIVES IN THE DISCOVERY OF MELATONIN

Professor of Dermatology and Chair of the Department of Dermatology at Yale University for 30 years, A. Lerner had a keen interest in pigmentation from his undergraduate years (2, 3). Inspired by the research of C. McCord and F. Allen, from 1927, who managed to lighten the color of amphibian tadpoles by adding bovine pineal powder in the water they were swimming in, Lerner and his associates set out to identify the unknown compound responsible for this change. This was of particular interest to them since they thought that the unknown substance was potentially involved in the pathogenesis of vitiligo. Over the course of four years, 200,000 bovine pineal glands weighing a little more than 100 kilograms, were delivered to their lab. Each 200-milligram gland yielded after dehydration, only 40 milligrams of tissue, giving them a minute quantity of material to work with. In 1958, they were finally able to extract 100 micrograms of the substance they were seeking (4).

They named the substance Melatonin, from the Greek word *melas (μέλας)*, meaning black/dark. The name meant to signify its effect on melanin, the pigment responsible for skin and hair color. Melatonin was able to blanch the amphibian skin by inducing the accumulation of melanin granules within the cells. However, their initial enthusiasm diminished when they realized that it

had no effect on the pigmentation of humans. Consequently, Lerner abandoned his interest in melatonin as a factor altering skin pigmentation. Before leaving the field though, he made one notable observation: both patients and himself would experience drowsiness and sleepiness after taking melatonin and that represents the first documentation of what later became recognized as melatonin's cardinal role (2, 5).

1.1.2 EVOLUTIONARY ROLE AND BIOLOGICAL SIGNIFICANCE

From an evolutionary perspective, melatonin is a highly conserved molecule, remaining structurally unchanged for nearly 2.5 billion years (6). Its original function in ancestral proteobacteria is considered to be that of an antioxidant and free radical scavenger, for the neutralization of the toxic byproducts of photosynthesis (7). For much of the twentieth century, the prevailing theories of evolutionary biology postulated that eukaryotic cells developed through mutation and natural selection. A few scientists proposed some alternative ideas. Among them, K. Mereschkowski, in 1910, who suggested that chloroplasts originated from cyanobacteria and introduced the theory of symbiosis, and I. Wallin, in 1927, who argued that mitochondria were once, independent bacteria (8). Nevertheless, their proposals were dismissed by the scientific community as speculative and unsubstantiated. In 1967, L. Sagan challenged the existing beliefs with a groundbreaking paper introducing the concept of endosymbiosis as an evolutionary model supported by evidence from cell biology, biochemistry and microbiology of that time (9). Though her ideas were initially met with skepticism, they become widely accepted as molecular and genetic evidence started accumulating and currently represent one of the most important paradigm shifts in evolutionary biology.

According to the endosymbiotic theory, mitochondria originated from ancestral aerobic bacteria, related to the modern α-proteobacteria, engulfed by the primitive eukaryotic cells, an estimated 2.5 billion years ago. That coincided with the Great Oxidation Event, which witnessed a rise of molecular oxygen in the atmosphere of the Earth, as a by-product of photosynthesis (10). This created an evolutionary pressure on the primitive cells which had to adapt to an oxidizing environment. Bacteria were initially engulfed by the primitive eukaryotes for their nutritional value, but a symbiotic relationship evolved, providing the bacterium with protection and nutrient supply, and the eukaryote with the machinery to efficiently utilize oxygen and a means to neutralize the free radicals generated in the process: melatonin (11). Current evidence for the

validity of the theory includes the fact that mitochondria have maintained their own DNA and possess the enzymes for melatonin synthesis, as well as genomic analyses which confirm homology between mitochondria and α-proteobacteria (9, 10, 12). An interesting fact is that melatonin synthesis in most bacteria, is regulated by light (13).

Melatonin's structure remained stable over the eons, but its repertoire expanded considerably. With evolutionary advancement, organisms increased both in complexity and cellularity, necessitating a means for the coordination of their functions and the attainment of homeostasis. We could well hypothesize that melatonin came in handy and was very conveniently used by a centrally producing organ, the pineal gland, for the orchestration of physiological processes. The emergence of specialized melatonin receptors further refined the signaling system, providing the opportunity for targeted actions.

1.2 THE PINEAL GLAND

A detailed description and analysis of the embryological, anatomical and histological characteristics of the pineal gland is beyond the scope of the present thesis. However, a brief description of each has been included in order to refresh the memory of the reader and aid in the understanding of melatonin physiology.

1.2.1 EMBRYOLOGICAL DEVELOPMENT

Being a neuroendocrine organ, the pineal primordium originates from neuroectoderm, with its morphological development commencing during the fourth week of gestation as an invagination of the caudal roof of the diencephalon. Although the gland is a single anatomical structure located in the midline, research shows that it develops from two distinct areas of the anterior embryonic neural plate which later fuse during the 20^{th} week of gestation, to create a single organ (14, 15). Small capillaries start appearing at its base, which will later give rise to the gland's blood supply.

What follows is a proliferative stage, characterized by the differentiation of pinealoblasts. The glandular stroma starts developing from the surrounding connective tissue, which penetrates the gland in a trabecular fashion (14). The neuronal connections of the gland start becoming established, with one interesting phenomenon: a nerve emerges which connects the pineal to the

posterior commissure of the brain, an area responsible for the coordination of eye movement and pupillary light reflex. The nerve has been shown to be present in human embryos and the name nervus pinealis has been suggested (16, 17). It is considered to be ontogenetically equivalent to the pineal nerve observed in amphibians, connecting a photosensitive area of their brain (also known as the "third eye") to the rest of the brain. The nervus pinealis disappears in humans, although the exact stage of gestation has not yet been determined (18).

The gland is not fully functional at birth. The fetus and neonate rely on maternally produced melatonin through the placenta or breast milk, respectively (19). The gland reaches its final size between the ages five and seven.

1.2.2 TOPOGRAPHICAL ANATOMY AND HISTOLOGICAL CHARACTERISTICS

The shape of the gland is conical, hence the name *konarion* (Greek for pinecone) that was given to it by Galen. It measures 6-10 mm in length, 5-6 mm in width and 1.5-4 mm in thickness. It weighs 150-250 mg (14). Its surrounding capsule is of conjunctival origin containing, among others, melanocytes. Connective tissue septa enter the gland, dividing it into lobules and providing pathways for the vascular and nervous structures (15).

In the adult brain, the pineal gland is located at the junction between the diencephalon and the mesencephalon, under the splenium of the corpus callosum. It is suspended in the pineal recess of the third ventricle by the pineal stalk which connects it to the habenular and posterior commissures (18) (Figure 1). The arterial supply comes from 1-3 small branches stemming from the medial posterior choroidal arteries. Unilateral vascularization is observed in 30% of cases. Venous drainage is subject to great anatomical variation, ultimately draining into the great cerebral vein of Galen (14).

There is experimental evidence indicating that the pineal is part of the circumventricular organs, lacking a blood-brain barrier (14, 17). MRI studies depict contrast enhancement in those organs, due to the presence of fenestrated capillaries. Nevertheless, enhancement of the pineal is considered by many to be secondary to the rich capillary network surrounding and entering the gland (20).

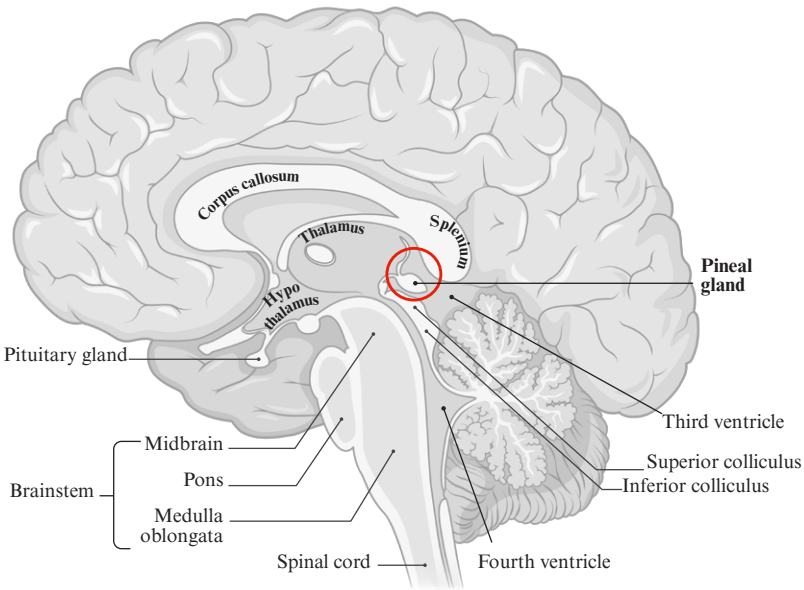

Figure 1: *Topographical relationships of the pineal gland (created with Biorender.com)*

The synthesis of melatonin is tightly regulated by external stimuli, making the gland's innervation a parameter of critical importance. Sympathetic innervation (nervus coronarii) originates from the superior cervical ganglion and constitutes the terminal part of a neural pathway that begins in retinal photoreceptors, and relays via the suprachiasmatic nucleus of the hypothalamus, where the master clock is located. Parasympathetic innervation comes from the sphenopalatine, trigeminal and otic ganglia (17). Finally, there is evidence of direct innervation from the brain parenchyma, mainly from the hypothalamus, via the pineal stalk. Disruption of the neural input renders the gland inactive (21).

The first to describe different cell types in the pineal parenchyma, in 1868 was G. Bizzozero, Professor of Pathology at the University of Turin, Italy (5). To date, five distinct cell types have been identified: pinealocytes, interstitial cells, perivascular phagocytes, neurons, and neuron-like cells. The pinealocytes synthesize and secrete melatonin. Interestingly enough, these cells express retinal antigens like opsin, recoverin and retinal S-antigen. The latter has been used as a tumor marker in pineal tumors (17).

There is a tendency for the accumulation of calcifications in the gland, which increases with age. They can be visualized radiologically and are referred to as “brain sand”(15). Identified in approximately 70% of the population, with a male predominance, they do not appear to affect the function of the gland (22).

1.3 BIOSYNTHESIS AND DEGRADATION OF MELATONIN

Melatonin is classified as an indole amine. It is synthesized across all biological kingdoms, though its biosynthetic pathway varies across animals, plants and bacteria (23). Its precursor molecule is the amino acid tryptophan, which is first converted to serotonin and then ultimately to melatonin. (Figure 2). J. Axelrod was the first to describe the pathway in the pinealocytes, in 1974 (24).

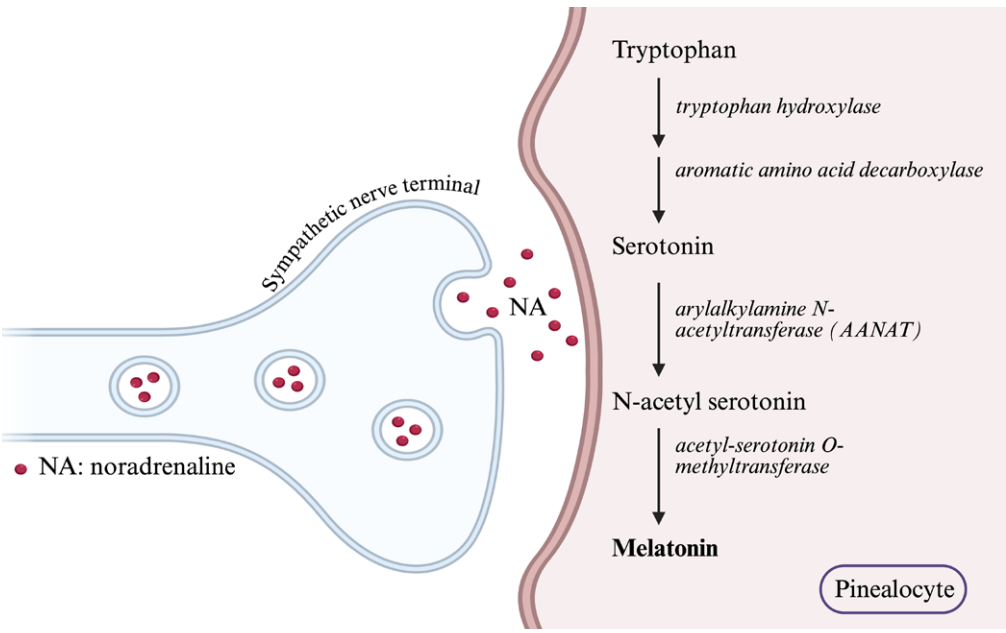

Figure 2: *Biosynthesis of melatonin (adapted from Axelrod, J.(24) , created with Biorender.com)*

Of all the enzymes involved in the biosynthesis of melatonin, arylalkylamine-N-acetyltransferase (AANAT) which catalyzes the rate-limiting step, is the most studied. The enzyme exhibits circadian rhythmicity, reflected by the circulating levels of melatonin. The process entails both up-regulation of AANAT synthesis, but also phosphorylation of the enzyme, rendering it up to seven times more active (23).

The regulation of the biosynthetic pathway is under the stringent control of the hypothalamus and regulated by the light/dark cycle. Since most vertebrates lack photoreceptors in their pineal gland, the required input is accomplished via an intricate neuronal pathway between the eyes and the gland, necessary for the conversion of a sensory input into a hormonal signal. The pathway begins at the retinal ganglion cells which convey photic information to the suprachiasmatic nucleus (SCN) of the hypothalamus, via the retinohypothalamic tract, embedded in the optic nerve. The SCN contains the "master clock", or central circadian pacemaker of the organism, which determines whether it is day or night, depending on the received retinal input. The signal is relayed to the paraventricular nucleus and, through the intermediolateral column of the spinal cord, to the superior cervical ganglion, from where post-ganglionic sympathetic fibers reach the pineal gland.

The retinal ganglion cells are intrinsically photosensitive, and contain melanopsin, a light-sensitive pigment that is maximally sensitive to the blue wavelength of light (460 – 480 nm). During the day, the signal is inhibitory, as light signals suppress the activity of the post-ganglionic sympathetic fibers, reducing their noradrenaline release into the gland, which in turn, decreases the production of melatonin. At night, the inhibitory signal is lifted, the release of noradrenaline to the gland increases and melatonin starts being synthesized and released (21, 23, 25) (Figure 3). For this reason, melatonin has been called the "hormone of darkness". Exposure to light at night (LAN) negatively impacts the production of melatonin, leading to circadian disruption (26).

Since pinealocytes lack storage capacity, following its biosynthesis, melatonin is released into the cerebrospinal fluid (CSF) of the third ventricle and the vascular circulation. Circulating melatonin originates almost entirely from the pineal gland, as is demonstrated by its minimal levels following pinealectomy. Serum melatonin concentration varies during the day, with peak plasma levels between 02:00 and 04:00, ranging from 80 pg/mL to 120 pg/mL, as opposed to daytime concentrations of 10 pg/mL to 20 pg/mL. Its half-life is

biexponential, characterized by an initial distribution phase of two minutes, followed by a secondary phase with a half-life of twenty minutes (27, 28).

The liver metabolizes more than 90% of the circulating melatonin, which is then excreted in the urine in the form of 6-sulfatoxymelatonin (28). The concentration of 6-sulfatoxymelatonin in the urine, mirrors quite accurately that of plasma concentration and can be used for the assessment of melatonin plasma fluctuations.

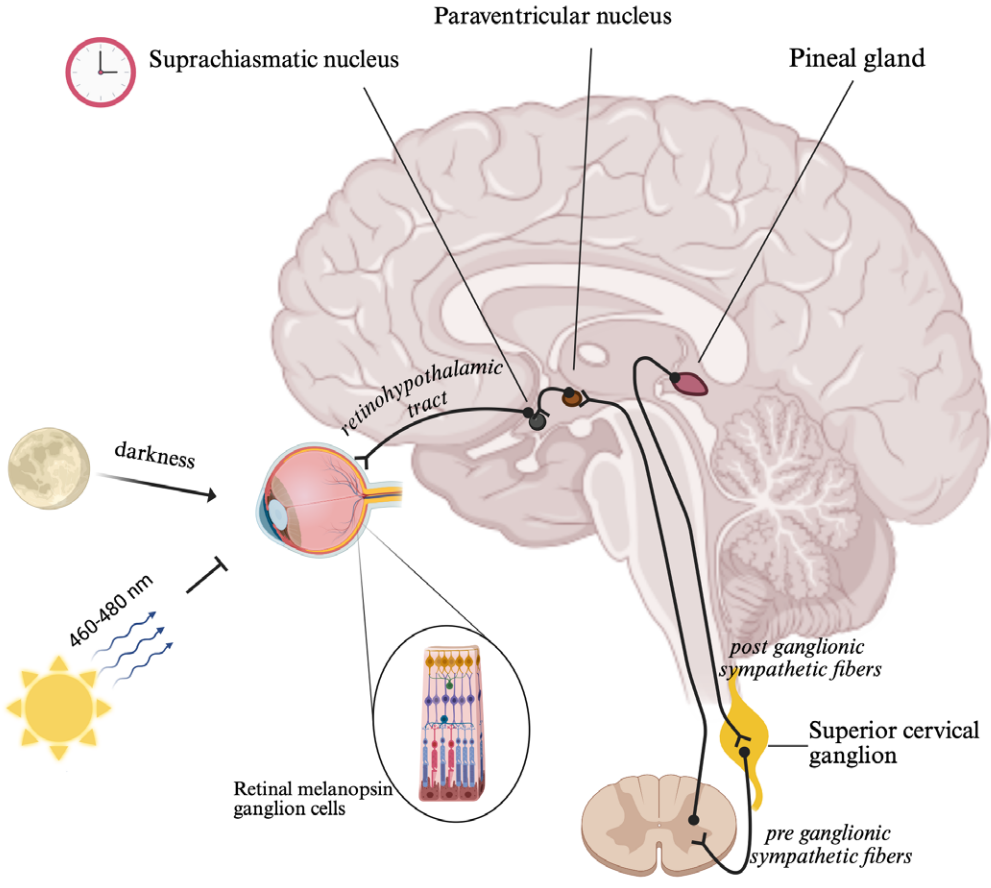

Figure 3: *Regulation of biosynthesis of melatonin (created with Biorender.com)*

As mentioned earlier, melatonin is not exclusively synthesized in the pineal gland, which is estimated to be responsible for approximately 5% of its total production (10). It has been successfully isolated in almost every human tissue and body fluid. Besides, the enzymes required for its synthesis have been isolated from various tissues and it is probable that it is produced in the matrix of mitochondria, serving its ancestral role, namely that of an antioxidant (29). Generally referred to as extrapineal melatonin, its synthesis is not light-dependent but rather inducible, dependent on tissue demands (30). Tissue concentrations higher than in plasma have been reported in, for example the gastrointestinal tract (up to 100 times higher than plasma), but this finding has been questioned by some researchers (30, 31). Body fluids containing high concentrations of melatonin include CSF, amniotic fluid, breast milk and bile. Taken together, while central production in the pineal sets and maintains circadian rhythmicity, local production helps conserve cellular homeostasis (32).

1.4 MELATONIN RECEPTORS AND SIGNALING MECHANISMS

The first mammalian melatonin receptor was cloned in 1994, followed by a second one in 1995 (33, 34). They were named MT1 and MT2, respectively. MT1 contains 350 amino acids, while MT2 contains 362. Their amino acid sequence is 60% identical and their coding genes reside on different chromosomes: chromosome 4q for MT1 and chromosome 11q for MT2 (35). Both are high-affinity receptors, meaning that binding of melatonin is possible at nM to pM concentrations, corresponding to the physiological concentrations measured in the brain and peripheral tissues (36).

Both are G protein-coupled receptors with seven transmembrane alpha helical domains. The amino terminal is located on the extracellular side. A characteristic feature of both is the existence of a pore, referred to as the “lateral channel”, which has been advocated as an entry gate for ligands. This agrees with the amphiphilic nature of melatonin, which is able to cross the lipid bilayer of the cell membrane (36). Activation of both receptors causes inactivation of adenylate cyclase with resulting reduction in cAMP formation and decreased Protein Kinase A activity. In turn, this reduces phosphorylation of cAMP-responsive element binding protein (CREB), a potent transcription factor, regulating gene expression (Figure 4). Pathways unique to MT1 include

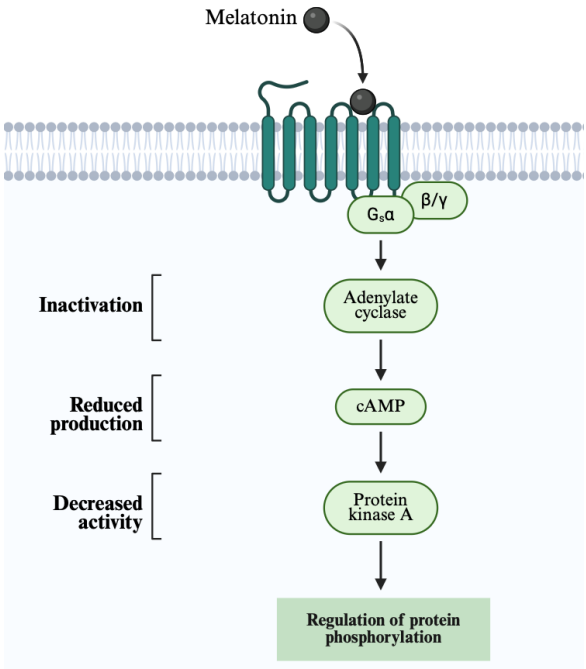

Figure 4: *Common MT1 and MT2 signaling pathway (adapted from Masana, M. (37), created with Biorender.com)*

the induction of phosphoinositide turnover and an increase in the intracellular calcium concentration. Likewise, MT2 produces inhibition of cGMP formation and an increase in Protein Kinase C (37). The responses evoked upon activation of the receptors, tend to be tissue specific.

Another notable characteristic of MT1 and MT2 is their ability to form dimers, either alone (homodimers) or with each other (heterodimers). The physiological significance of this can be deduced by the co-existence of the receptors in several tissues and is still under investigation. Furthermore, MT1/MT2 heterodimers appear to have a different pharmacological profile than MT2 homodimers (38).

The distribution of the receptors varies. MT1 is principally expressed in the cardiovascular system (heart and major blood vessels), immune cells, retina, testes, ovary, skin, liver, kidney, adrenal cortex, placenta, breast, pancreas, and spleen. In the brain, it is predominantly found in the hypothalamus, cerebellum, hippocampus, substantia nigra and central tegmental area. The MT2 receptor

is mainly expressed in blood vessels, immune cells, retina, kidney, gastrointestinal tract, adipose tissue and skin. In the brain, it is mostly localized in the hypothalamus, suprachiasmatic nucleus and the pituitary (39).

Receptor polymorphism has been recognized in both receptors. Several variants in MT2 have been associated with pancreatic beta-cell dysfunction, elevated fasting plasma glucose, increased risk for type 2 diabetes and diabetes of pregnancy (40). Several studies have tried to investigate primarily MT1, but also MT2 receptor polymorphism with inconclusive and sometimes contradictory results. A Korean study associated certain variants of the gene coding for MT1 with increased breast cancer risk, with a further risk increase when interacting with night shift work. Their results were not replicated in similar western studies and ethnic variations have been proposed as a potential contributing factor (41, 42). Comparable investigations have been conducted for prostate, hepatocellular and urothelial carcinomas and for oral cancer. To date, the obtained results, remain non-definitive (38).

An additional membrane receptor belonging to the melatonin receptor subfamily, the GPR50 receptor was cloned in 1996 (36). It has a 45% homology to MT1 and MT2, but it does not bind to melatonin and remains, to date, an orphan receptor, having no known ligand. It is expressed in areas of the hypothalamus related to body weight control and metabolism. GPR50 has the capacity to form homodimers, but also heterodimers with MT1 and MT2. Heterodimerization of GPR50 with MT2 does not alter its function; on the contrary, heterodimerization with MT1 renders the latter inactive (43). Sequence variants of the GRP50 have been related to higher fasting circulating triglycerides (44).

Besides the above-mentioned receptors, a third receptor, originally called ML2, is currently called MT3. Contrary to MT1 and MT2, it exhibits low affinity for melatonin, and its function is temperature-dependent (45). Research has demonstrated that MT3 is the cytosolic enzyme quinone reductase 2, with which it shares a 95% homology (39, 45). An in vitro study demonstrated increase in chemotherapy-induced apoptosis in HT-29 and HeLa cancer cell lines with the addition of melatonin, the effect was mediated by MT3 (46).

Melatonin is also capable of binding directly to nuclear receptors, and studies have focused particularly on the superfamily of retinoic acid-related orphan receptors (ROR). Due to its amphiphilic nature, melatonin readily crosses

cellular membranes and enters the nucleus. Nevertheless, the available evidence to date, regarding melatonin as a ligand to ROR, remains incomplete (47).

1.5 BIOLOGICAL ACTIONS OF MELATONIN

Melatonin exerts multiple effects on the organism, that are either receptor-dependent or receptor independent. What follows is a brief description of the most important of them, on both systemic and cellular levels. The oncostatic effects are discussed separately (Part 1.7)

1.5.1 SYSTEMIC EFFECTS

1.5.1.1 CIRCADIAN RHYTHMICITY

The principal function of melatonin is the regulation of the circadian rhythm. This is accomplished by its effects on the SCN of the hypothalamus, which hosts the master clock of the organism. Being situated right above the optic chiasm and in close proximity to the supraoptic recess of the third ventricle, its position is ideal for receiving direct input from the retina and the release of melatonin by the pineal gland. The SCN has its own intrinsic rhythm which approximates 25 hours and is not in synchrony with the normal 24h period, requiring a means for the synchronization between the two (26). Central clock alignment relies on environmental cues collectively known as zeitgebers (German for “time-givers”) and involve the light-dark cycle, feeding times, temperature alternations, but also social interactions. The fluctuating concentrations of melatonin in the CSF, reflecting the photoperiod, provide feedback to the SCN for the resetting of the rhythm in accordance with geophysical time (48). Circadian control is an evolutionary conserved function regulating homeostatic pathways responsible for survival. These include the sleep/wake cycle, feeding rhythms and a variety of endocrine and immune functions. Information concerning the photoperiod is of critical importance to seasonal breeders, but largely obsolete to humans. The central clock is also responsible for the synchronization of peripheral clocks, found in almost all peripheral tissues, which also rely on external cues, such as temperature and food availability (49) (Figure 5).

The exact role of melatonin in sleep induction and regulation has not been fully delineated. A widely accepted conjecture is that it exerts an inhibitory effect

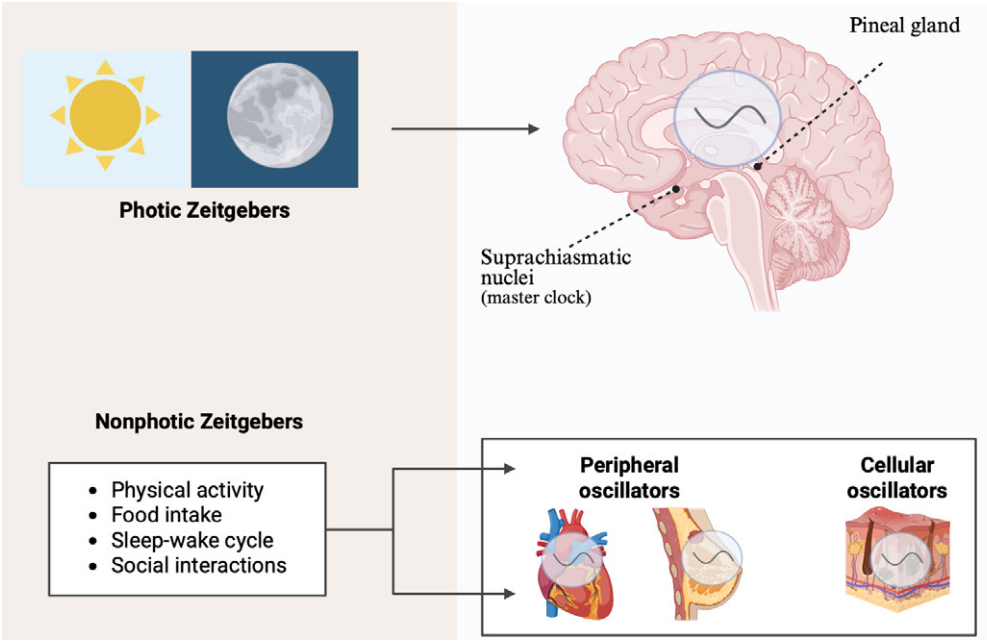

Figure 5: *Influence of Zeitgebers on circadian coordination (created with Biorender.com)*

on the SCN (50). Though not a hypnagogue itself, melatonin is involved in the initiation of sleep through its direct effects on the hypothalamus, and sleep maintenance through its effects on the thalamus (51, 52). Existing but limited research suggests that it is also a bioprecursor of sleep-promoting metabolites (53).

1.5.1.2 ENDOCRINE CONTROL

Historical observations have suggested a connection between the pineal and the endocrine system, giving rise to numerous research projects investigating the hypothesis that the gland has an inhibitory action on the gonads. A case report was published in 1898, presenting a boy with precocious puberty and a tumor in the area of the pineal gland (54). What was presumed until the 1950s was that precocious puberty was the result of decreased pineal function due to disease in the surrounding structures and that delayed sexual maturation was due to increased pineal activity from tumors in the gland itself (1).

Melatonin production peaks during childhood, demonstrating a substantial decrease in the beginning of adolescence, coinciding with the rise in pituitary

hormones. Human studies indicate a decrease in melatonin secretion during puberty. This raises the question of whether the relationship is causal and in which direction. Animal studies have shown that melatonin supplementation delays puberty, whereas pinealectomy has the opposite effect (55). Concluding, it is not yet known what the exact relationship between melatonin and the reproductive hormones is, but melatonin appears to be one of the permissive factors for the initiation of puberty (56).

1.5.1.3 IMMUNOMODULATION

Melatonin is increasingly being recognized as an important regulator in immune functioning in both innate and adaptive immune responses. The interplay between melatonin and the immune system is further supported by the existence of melatonin receptors in immune organs and cells (57). It stimulates the proliferation and activity of the main cellular components of innate immunity: natural killer cells, monocytes, macrophages and neutrophils (58). It supports the immune defense through the enhancement of the production of cytokines (IL-2, IL-6, γ-interferon) by acting on helper-T cells.

The above-mentioned functions of melatonin, together with its antioxidant and tumor-suppressing effects constitute it a candidate for bolstering immune surveillance and effectiveness, as well as restoring LAN-induced immunity impairment (59). Caution should be taken however in auto-immune disorders, where animal models have demonstrated inconclusive and even negative effects of melatonin in the progress of the disease (60)

1.5.1.4 ADDITIONAL FUNCTIONS

Melatonin's effects have been extensively studied in various physiological processes, among which two deserve further commenting. Its neuroprotective effects have been demonstrated in animal models of induced stroke and of Alzheimer's and Parkinson's disease and are thought to be mediated by its antioxidant and free radical scavenging properties as well as its ability to preserve the blood-brain barrier and assist in the functions of the glymphatic system (51, 61). In addition, the cardioprotective role of melatonin has been demonstrated in pinealectomy-induced hypertension models and cardiac infarct studies. At the vascular level, it enhances nitric oxide production promoting vasodilation (51, 60).

1.5.2 CELLULAR AND MOLECULAR EFFECTS

1.5.2.1 ANTIOXIDANT AND FREE RADICAL SCAVENGER

The ancestral role of melatonin was that of an antioxidant and free radical scavenger, protecting the primitive cells from the emerging oxidative atmosphere and the by-products of photosynthesis. That role has been maintained through evolution and is still called upon at a local level. With the majority of its production occurring outside the pineal, mainly in the skin and the gastrointestinal tract, it remains in situ for local use (62). During the detoxification process, which usually occurs by electron donation, the resulting derivatives can be as effective as melatonin itself resulting in a cascade of free radical scavenging (63). Moreover, it maintains the ability to synergistically operate alongside other known antioxidants such as vitamins C and E and glutathione. Due to its amphiphilic nature, it is able to operate in both lipid and aqueous environments, achieving a more potent antioxidant capacity than the lipid-soluble vitamin E or the water-soluble vitamin C (64). Evidence for the capability of melatonin in assisting cells to surpass oxidative stress comes from studies on ischemia-reperfusion injury and organ transplantation (62).

1.5.2.2 MITOCHONDRIAL PROTECTION

Primarily because of its ability as a free scavenger, melatonin is particularly important in the preservation of the mitochondrial cell membrane, from the free radicals produced during oxidative metabolism (65). This constitutes a logical deduction if one considers the evolutionary origin of the mitochondria. The enzymes required for its synthesis have been identified in the inner and outer membranes, as well as the inter-membrane space (7). In addition, melatonin can reduce free radical production in two different ways, a process also called free radical avoidance. The first way is by accelerating the flow of electrons through the electron transport chain, thus reducing electron leakage and the generation of reactive oxygen species. The second is by lowering the mitochondrial membrane potential, reducing again electron leakage (66). Research on neuronal cells has also demonstrated its ability to up-regulate other antioxidant enzymes (67). Even though passive diffusion is an explanation frequently used to explain the presence of melatonin inside any organelle, there seems to be evidence of two transport systems which assist its transport through membranes (68).

1.5.3 CLINICAL IMPLICATIONS AND THERAPEUTIC PROSPECTS

1.5.3.1 ABERRANT MELATONIN PRODUCTION

Hypomelatoninemia is characterized by reduced melatonin production or nocturnal peak value, compared to the expected for the age of the individual. It can be primary due to factors affecting the gland or its innervation, e.g. tumors compressing or destroying the gland parenchyma. In vivo studies involving compression of the pineal have shown such a decrease (69, 70). Alternatively, they could be secondary due to LAN. The coexistence of precocious puberty formerly described in association with pineal tumors is nowadays attributed to the production of human chorionic gonadotropin by germ cell tumors in the pineal area, in particular teratomas (71, 72).

Hypermelatoninemia is defined as the hyperproduction of melatonin due to true hormone-producing pineal tumors (72, 73).

1.5.3.2 CHRONODISRUPTION

The use of artificial light on a worldwide scale, has caused the advent of light pollution affecting both social and biological processes. Exposure to light at night includes mainly the utilization of artificial lighting and electronic devices and night shift work. Continuous exposure to light has been shown to cause hormonal, reproductive and behavioral changes that are at least partly attributed to circadian disruption (74). Furthermore, animal experiments indicate that continuous light exposure accelerates the ageing process, decreases life span, and enhances the spontaneous appearance of tumors (75, 76).

The concept of chronodisruption has emerged, defined as a disturbance in the alignment between the internal biological processes and the external environmental cues (77). The foremost external chronodisruptor is LAN which can cause both a phase-shift in melatonin production and a suppression of its synthesis and release. Blue light (460-480 nm) particularly suppresses its synthesis and is also linked to hormonal dysregulation. Nevertheless, the potential effect of other factors such as feeding patterns, social and environmental cues should not be overlooked.

Research on chronodisruption has focused mainly, though not exclusively, on its effect on carcinogenesis. A recent meta-analysis demonstrated increased risk for breast, thyroid and prostate cancers under LAN conditions, and also

emphasized the importance of DNA damage and genomic instability among the underlying factors (74). Along the same lines, LAN has been shown to evoke a state of chronic low-grade inflammation, impair proper functioning of the immune system and predispose to obesity and metabolic syndrome (42). Along the same lines, a review article on circadian clock dysregulation summarizes data from current research, showing the effect of chronodisruption on cancer initiation, progression, metabolic reprogramming and response to therapy (78).

1.5.3.3 JET LAG

Formerly described as circadian desynchrony, jet lag is a type of chronodisruption that occurs when traveling across multiple time zones, due to the misalignment between the endogenous circadian clock and the external light-dark cycle. Clinical manifestations include sleep disturbance, fatigue, cognitive impairment and gastrointestinal symptoms. The severity of the symptoms depends on the number of time zones crossed and the direction of the travel, with eastward travelling being worse. Melatonin administration has been used as a therapeutic agent for jet lag to facilitate circadian phase shifting, sleep onset and adaptation to the new time zone (79, 80). A Cochrane meta-analysis of ten randomized controlled trials concluded that melatonin is highly effective in the prevention and mitigation of jet lag. Its use is primarily recommended for adult travelers travelling across five or more time zones, especially eastwards, and for those with a history of jet lag. Those travelling fewer time zones may also derive benefit. Short-term dosage appeared to be safe, but the authors recommended more systematic investigation of the pharmacological properties of melatonin and its interaction with other drugs (81).

1.5.3.4 SLEEP DISORDERS

Sleep disorders encompass a diverse group of diseases from insomnia to seasonal affective disorder to obstructive sleep apnea syndromes. Melatonin has been studied and used extensively in the treatment of some of them. If given at the appropriate time (late in the evening), and at low doses, it is able to shift the circadian phase (82). An attractive aspect concerning its use in the adult population is the treatment of age-related insomnia. The production of melatonin decreases with age and is manifested as difficulty in induction and/or maintenance of sleep (83). Contrary to the general belief, the elderly need roughly the same amount of sleep as the younger adults, and administration of melatonin has been reported to have beneficial effects (84).

1.5.3.5 MELATONIN AS A PRESCRIPTION DRUG/SUPPLEMENT

Melatonin is available as an over-the counter dietary supplement in many countries, whereas it is available only by prescription in some others, Sweden included. Among adults, its principal indication is for the management of sleep disturbances and jet lag. A number of studies have evaluated the adverse events associated with melatonin use. The two systematic reviews that have been published up to date (85, 86) concluded that short-term administration is generally well-tolerated. The most frequently reported adverse events included somnolence, headache, fatigue and transient impairment of psychomotor and cognitive functions, particularly when melatonin was taken during the daytime i.e. not in sync with the circadian cycle.

Reductions in heart rate and blood pressure have been reported, but it is unclear if they consist true side effects or represent interactions with concomitant antihypertensive therapy. Additional reported effects include alterations and endocrine functions and glucose metabolism. To date, no studies exist on its long-term use (87, 88). Of particular concern is its use by older adults, who exhibit delayed melatonin metabolism and risk having elevated plasma concentrations (89). Administration of melatonin is not recommended during pregnancy and lactation.

An issue worth commenting upon is that there is no overall consensus regarding the dosage. The standard advice is to start with the lowest dose and successively increase with demand. Doses of 0.1-0.3 mg per day bring about near physiological plasma concentrations and are suitable for circadian synchronization. Up to 5 mg per day have been used for the management of sleep disorders (90).

In October 2020, a small melatonin blister pack became available in Sweden for purchase over the counter, for the short-term management of jet lag. Larger packs still require a prescription. The official therapeutic indications include treatment of sleeping disorders in children and adolescents (ages 6-17) with ADHD, when behavioral strategies have failed, and short-term management of jet lag.

1.6 BREAST CANCER

Breast cancer is the most common malignancy in women, and from 2020, it is the most commonly diagnosed cancer (91). What's more is its increasing incidence, irrespective of income level, due to global ageing and growth of the population. Incidence is higher in high-income areas, reflecting both lifestyle factors and screening availability, where is usually diagnosed at an early stage, leading, together with the availability of improved treatment options, to favorable prognosis.

1.6.1 RISK FACTORS

Being a multi-factorial disease, as most cancers are, breast carcinogenesis advances on the combination of multiple risk factors, which can be divided into non-modifiable and modifiable. Non-modifiable risk factors include female sex, older age, ethnicity, family history and genetic susceptibility. Factors that are largely inherent, such as early menarche and late menopause as well as increased breast density are also associated with increased risk. Mutations in high-penetrance genes such as *BRCA1* and *BRCA2* (average cumulative risk by 80 years of age: 72% and 69%, respectively), and *TP53*, significantly increase lifetime risk, while mutations in moderate and low penetrance genes (e.g. *PALB2*, *CHEK2* and *ATM*) can also impact. A personal medical history of prior chest irradiation, especially at a young age is associated with an elevated risk. A history of proliferative breast lesions with or without atypia have also been found to confer an increased risk (92-94). Modifiable risk factors include behaviors and/or exposures that can be adjusted or removed through individual or public interventions. Hormone replacement therapy and to a lesser extent oral contraceptives can increase the risk by constantly stimulating the proliferation breast tissue. Obesity, particularly in post-menopausal women, metabolic dysregulation and lack of physical activity are increasingly being recognized as risk factors. Night shift work has been associated with increased risk. Smoking, alcohol consumption, vitamin D deficiency, environmental pollutants such as organochlorides, have all been implicated in risk escalation. Advanced age of first full pregnancy, number of children and lack of breastfeeding are also modifiable risk factors (93).

1.6.2 CLASSIFICATION

At a molecular level, breast cancer is a biologically heterogeneous disease. All breast cancers arise in the terminal duct-lobular units of the collecting ducts. Their histological and molecular characteristics determine to a great extent the

therapeutic approach. Invasive ductal carcinoma (currently referred to as "no special type") and invasive lobular carcinoma are the most common subtypes, accounting for approximately 75% and 15%, respectively (95). In 2000, C. Perou proposed the classification of breast cancer based on their gene expression patterns (96). At least four intrinsic subtypes have been recognized: luminal A and B, HER2-enriched, and basal-like. The luminal subtypes are the most common and are so called because their gene expression reminds that of the luminal epithelial cells of the normal breast. They express estrogen (ER) and progesterone (PR) receptors and can be distinguished into two subtypes. Luminal A tumors tend to exhibit low Ki67 proliferation index and are HER2 negative. Conversely, luminal B tumors are more proliferative, are sometimes HER2 enriched and have a lower expression of PR. The rest of the subtypes do not express hormone receptors. HER2 enriched, expresses HER2 and a high proliferation. Basal-like tumors, express the basal cluster of genes and are molecularly more similar with tumors originating from the basal layer of the epidermis and epithelial ovarian cancer. Women with *BRCA1* mutation have basal-like tumors up to 80% of the time. An additional subtype that has been identified is Claudin-low, that is quite similar to the basal-like, but does not express the same proliferation (93, 96).

In clinical praxis, we use the surrogate intrinsic subtypes based on histology, immunohistochemical assessment of ER, PR, HER2 and the proliferation marker Ki67, in accordance to St:Gallen international expert consensus (97). Cancers expressing ER and/or PR are "hormone receptor positive", while those that do not express ER, PR, and HER2 are "triple negative" (Figure 6). Over the past 15 years, this heterogeneity has changed treatment strategies, shifting away from extensive surgical resections, towards management guided by the biological characteristics of the tumor. This shift in perspective has been facilitated by the development of targeted therapies and has guided de-escalation tactics which aim at reducing treatment toxicity, without reducing its efficacy. Nevertheless, features such as locoregional tumor burden and metastatic dissemination continue to play a role in decision-making (92).

1.6.3 TREATMENT OF BREAST CANCER

Early breast cancer, confined to the breast and axillary region, is deemed curable. Treatment modalities include surgery, chemotherapy, endocrine therapy, radiation therapy, targeted therapies and immunotherapy. This constitutes a diverse toolset at the disposal of contemporary medical professionals in order to tailor therapy to the biological characteristics of the

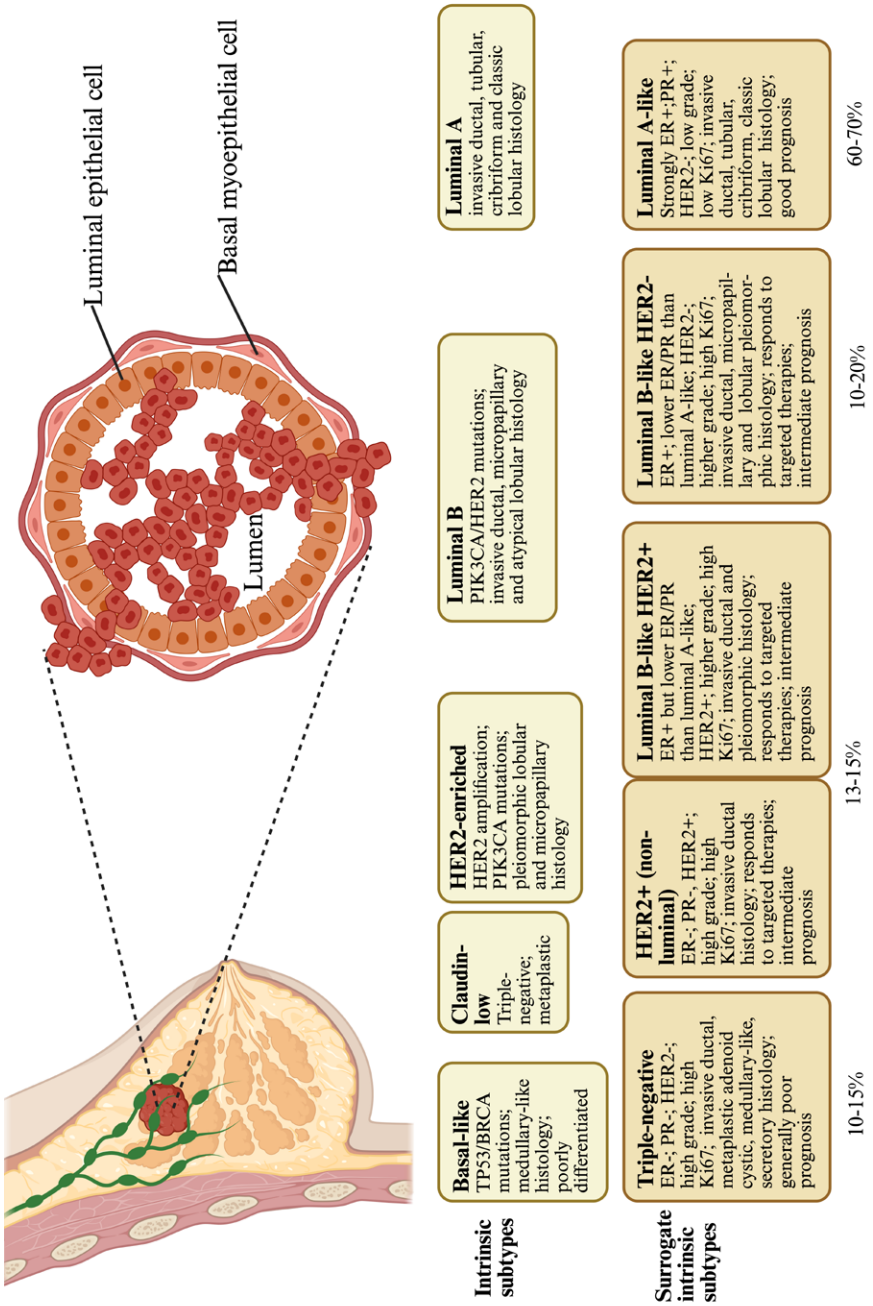

Figure 6: *Surrogate and intrinsic subtypes of breast cancer (adapted from Harbeck, N. (92),created with Biorender.com)*

tumor and the biological status, and to the extent that this is possible, the patient's choices.

1.6.3.1 SURGERY

Surgery continues to be the cornerstone of therapy, for the removal of the tumor and axillary staging with sentinel node biopsy, or in selected patients, lymph node dissection. Radical mastectomy, based on W. Halsted's theory that breast cancer is a locoregional disease, involved the en block removal of the breast, pectoralis major and minor, and axillary lymph nodes, and had been the standard of treatment until the 1970's. The procedure was associated with considerable morbidity and had limited impact on long-term survival, despite achieving local control. The National Surgical Adjuvant Breast and Bowel Project (NASBP) B-04 trial randomized patients with clinical negative nodes to radical mastectomy, modified radical mastectomy, or simple mastectomy with node irradiation and surgical intervention in cases of clinical node involvement. No differences in survival were found after 25 years of follow-up. This landmark trial led to the abandonment of modified radical mastectomy (93). Breast conservation seemed like the next logical step and after a series of non-randomized and randomized trials comparing modified radical mastectomy to breast conserving surgery and irradiation, breast conservation is now the standard of care for patients with early breast cancer (98). Patients who still require a mastectomy are those with unfavorable tumor/breast volume ratio, those with extensive calcifications and those with inflammatory breast cancer (99). Concurrently, surgery in the axilla moved from nodal dissection to sentinel lymph node biopsy, which accurately stages the axilla, while reducing the morbidity of extensive and sometimes unnecessary surgery in that area.

The surgical management of breast cancer has been guided by the principle of "less is more" during the past decades, with the surgeons removing smaller parts of the breast tissue, especially after the consensus on margins as "no tumor on ink" (97). Several attempts have been made to refrain from surgery of the breast after neoadjuvant therapy with clinical compete response. Early trials compared radiotherapy to breast surgery, and the follow-up showed higher recurrence in the radiotherapy arms. This led to the refinement of patient selection with the use of ultrasound-guided vacuum-assisted biopsies to confirm pathologic response. It appears thar omission of breast surgery could represent an option for a highly selected group of patients, deemed as "exceptional responders" (100-102).

1.6.3.2 ENDOCRINE THERAPY

Estrogen exerts a proliferative effect on normal breast tissue by stimulating division of the epithelial cells, but the effect is augmented on breast cancer cells. The stimulatory effect of estrogens in driving breast carcinogenesis, is both through systemic circulating estrogens and their local production in the tumor microenvironment. Their concentration in breast tumors has been found to be higher in the tumor area than in plasma or normal breast tissue (103). By binding to their receptor, they can activate gene expression through binding to the estrogen response element (ERE) in the promoter region of specific genes. In addition, they can directly interact with growth factor receptors, up regulating the expression of genes pertaining to cellular proliferation and survival. As a consequence, reducing plasma estrogen concentration, or hindering their effect on the mammary gland by blocking their receptors, plays a pivotal role in the management of hormone-sensitive breast cancer (92, 103).

The first hormonal treatment was surgical and dates back to 1896 when G. Beatson performed the first oophorectomy on a patient with advanced breast cancer, only to witness a remarkable response afterwards. The second patient did not respond but rather progressed. However, at that time, there was no knowledge of estrogen receptors which were discovered some 70 years later (104). Before the advent of pharmacological agents, hormonal treatment for breast cancer entailed the surgical removal or irradiation of the ovaries. Both are still practiced today, but ovarian function suppression can nowadays be achieved through pharmacological means.

Endocrine therapy was the primary targeted therapy for cancer. Its targets include the ER, present in 60% of premenopausal and 75% of postmenopausal breast cancers, or the biosynthetic pathway of estrogens. (93) The pharmacological substances used can be classified as selective estrogen receptor modulators (SERMs) or selective estrogen enzyme modulators (SEEMs). Tamoxifen is the most commonly prescribed SERM, acting by competitively inhibiting estrogen binding to ER and exerts its oncostatic effects by inhibiting the estrogen-mediated transcriptional activity, causing cell cycle arrest and inducing apoptosis (105, 106). It is used in the neo-adjuvant, adjuvant and metastatic settings, and has been evaluated as a risk reduction agent in high-risk populations (107). In early, ER positive breast cancer, it reduces the risk of recurrence and increases disease free survival. It remains the first-line choice for premenopausal patients with hormone-sensitive breast cancers.

Aromatase is an enzyme responsible for the conversion of adrenal androgens to estrogens, which constitutes the principal route of estrogen production in post-menopausal women. It is present primarily in adipose tissue, brain, bone and the breast. The third-generation aromatase inhibitors, which include anastrozole, letrozole and exemestane, constitute the principal adjuvant endocrine treatment in postmenopausal patients with ER positive breast cancer. They act by inhibiting aromatase and are collectively classified as SEEMs. Their superiority over tamoxifen has been demonstrated in several trials and they may be used as initial therapy, or sequentially, in combination with tamoxifen, for the usual 5-year or extended treatment (108).

1.6.3.3 RADIATION THERAPY

Breast conserving surgery without breast irradiation is associated with increased rates of locoregional recurrence and therefore, adjuvant radiotherapy is currently a standard component in breast conservation. The primary objective is to eradicate residual microscopic disease, thereby reducing both the risk of local recurrence and the risk of death from breast cancer. The effect is more pronounced in younger patients. Boost irradiation gives an additional benefit. Post-mastectomy radiation therapy also improves locoregional control and reduces breast cancer mortality in selected patients with high risk of locoregional recurrence (adverse tumor biology, axillary metastasis, young age). Improved techniques have helped reduce the exposure of surrounding tissues such as the heart and the lungs, decreasing the risk of damage (93). The omission of radiation therapy has been investigated primarily in women aged 60 years or older, with ER positive, low-grade tumors, without axillary metastases. The results show better locoregional control in the radiation arm, but without survival benefit (99). The trials assume that enrolled patients are receiving endocrine therapy. This must be taken into consideration before making the decision to omit adjuvant radiation therapy, particularly because most patients report better quality of life post-radiation compared to endocrine treatment.

1.6.3.4 CHEMOTHERAPY

Chemotherapy plays an important role in the treatment of breast cancer, by targeting micrometastatic disease and reducing breast cancer mortality by approximately one third (92). It can be administered before or after surgery, with equivalent effects on outcomes, as demonstrated by the NSABP-B18 trial (109). Administration in the neoadjuvant setting has the additional advantage of downstaging the tumor allowing breast conservation. Chemotherapy is administered in selected patients with ER positive breast cancer, in patients

with HER2 positive tumors in combination with anti-HER2 therapy, and remains the mainstay of treatment for triple negative breast cancer (92, 99).

1.6.3.5 ANTI-HER2 THERAPY

HER2 amplification is present in approximately 15% of breast cancers, which are characterized by high proliferation and adverse clinical outcomes. The activation of HER2 receptor instigates increased proliferation, invasion and angiogenesis (93). The prognosis of HER2 positive breast cancer has improved significantly since the introduction of anti-HER2 antibody therapy, which is administered in combination with chemotherapy, either before, or after surgery. Administration of antibody-drug conjugates containing a combination of anti-HER2 antibody with a potent chemotherapeutic agent has expanded treatment opportunities and the potential of treatment de-escalation (110). Additional considerations include the de-escalation or even omission of chemotherapy in selected patients with HER2 positive tumors. In cases where the tumors are also hormone receptor positive, there is the potential for endocrine therapy to replace chemotherapy, while maintaining HER2 control with trastuzumab and pertuzumab (100).

1.6.3.6 CDK4/6 INHIBITORS

Cyclins are a family of proteins that regulate the progression through the different stages of the cell cycle by activating cyclin-dependent kinases (CDKs). Dysregulation of the Cyclin D1-CDK4/6 axis appears to be an early step in breast cancer pathogenesis. Their inhibition leads to cell cycle arrest. In luminal breast cancers, the combination of CDK4/6 inhibitors with aromatase inhibitors has demonstrated an extension in progression-free survival in patients with metastatic disease (111). In the adjuvant setting, two trials have demonstrated improved disease-free survival with the use of ribociclib or abemaciclib in combination with endocrine therapy (112). However, the development of resistance to CDK4/6 inhibitors represents a challenge.

1.7 MELATONIN AND THE HALLMARKS OF CANCER

Cancer is a multi-factorial disease necessitating a series of molecular and cellular changes before and during its initiation and progression. In their iconic article from 2000, “The Hallmarks of Cancer”, D. Hanahan and R. Weinberg proposed six distinctive capabilities, critical for the malignant transformation of the cells (113). They later expanded them to eight (114), and subsequently introduced one more (115). A total of five enabling factors were also introduced, addressing phenotypic characteristics that enable the acquisition and activation of the hallmarks during carcinogenesis and progression. In the latest publication, senescent cells have been replaced by innervation (116) (Figure 7). Accumulating evidence suggests that melatonin exerts inhibitory effects on both the hallmarks and the enabling factors. The examples and bibliographic reference provided, relate mainly to breast cancer.

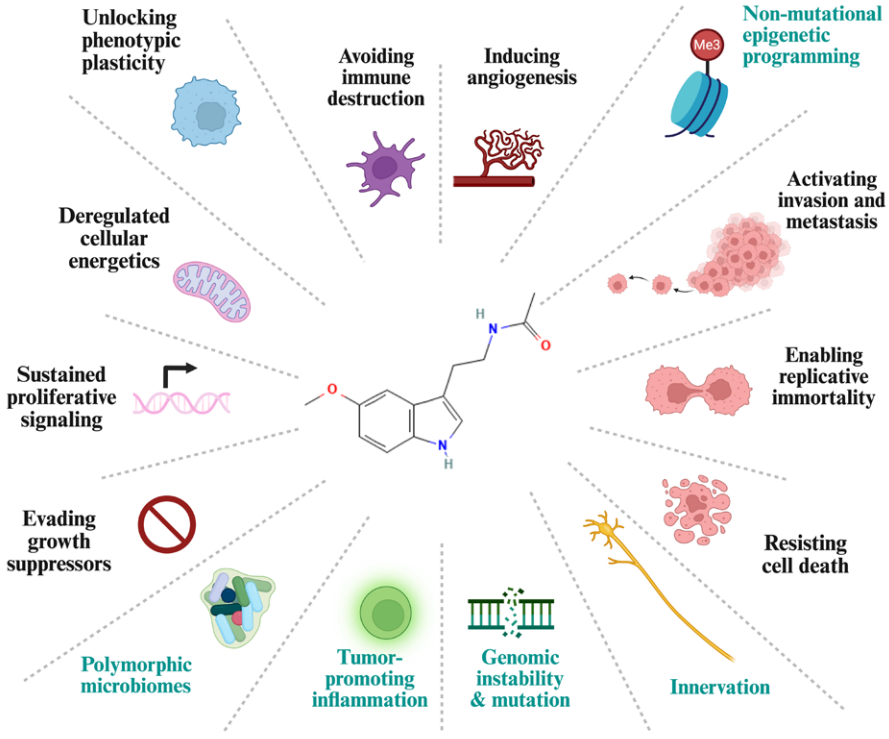

Figure 7: *The hallmarks (black) and enabling characteristics (green) of cancer (adapted from Hanahan, D.(116), created with Biorender.com)*

1.7.1 HALLMARKS

1.7.1.1 SUSTAINING PROLIFERATIVE SIGNALING

Cell division is under the stringent control of a complex network involving oncogenes, tumor suppressor genes and cell cycle checkpoints. Proliferation of normal cells necessitates growth signals originating from adjacent cells and their microenvironment. In cancer, this dependence is disrupted, leading to uninhibited cellular proliferation since malignant cells rely on their own signals for the (uncontrolled) regulation of their division. A few examples of this include: The PI3K/Akt pathway, shown to be inhibited by melatonin in both MDA-MB-361 and MCF-7 breast cancer cell lines (117, 118). Cyclin-dependent kinases (CDKs), critical components for cell division, the transcription of which has been shown to be inhibited in various types of cancers, including breast, after treatment with melatonin (119).

1.7.1.2 EVADING GROWTH SUPPRESSORS

Mutations in tumor suppressor genes enable cancer cells to evade programmed cell death. Tumor protein 53 (*TP53*) is an exemplary tumor suppressor gene, transducing information originating from within the cell, pertaining to intra-cellular stress, genome damage, oxygen and nutrient availability. It operates as a regulator of cellular cascades that ultimately determine growth arrest, apoptosis, senescence or DNA repair. Melatonin has been found to increase the expression of *TP53* in MCF-7 cells (120). It also activates the *TP53*-dependent arrest of the cell cycle, in CMF-7 breast cancer cell lines, reducing their ability to proliferate and build colonies (121). Interestingly enough, blocking melatonin receptors in those cells, impairs melatonin's inhibition of cellular proliferation (122).

1.7.1.3 ACTIVATING INVASION AND METASTASIS

The process of tissue invasion and metastasis entails changes in the shape of malignant cells and their attachments with other cells and the extracellular matrix (ECM) (114). Loss of E-cadherin, an important component of adherence junctions, is a well-studied event in the invasion-metastasis cascade. Melatonin has been shown to up-regulate the expression of E-cadherin in CMF-7 breast cancer cell lines (123). Likewise for the expression of integrin, a glycoprotein that helps anchor the cytoskeleton to the ECM (59, 119). Epithelial-mesenchymal transition (EMT) is a process through which epithelial cells acquire a mesenchymal phenotype with amplified migratory capacity, essential for the dissemination of cancerous cells. Studies in breast cancer stem

cells and MCF-7/HER2 cells indicate that treatment with melatonin inhibits EMT, reducing their capacity for invasion (124, 125).

1.7.1.4 ENABLING REPLICATIVE IMMORTALITY

The majority of tumor cells that are used in research are immortalized, with a limitless replicative capacity. Normal cells in culture are usually capable of 60-70 divisions, and it is the end part of the chromosomes, known as the telomere, that keeps the score. Telomerase is a nuclear enzyme responsible for the elongation of telomeres during cell replication, restoring their length and maintaining chromosomal stability. It is activated in 85%-90% of cancers (113). Inhibition of telomerase activity by melatonin has been demonstrated both in vivo and in vitro (126). Downregulation of CDKs, as discussed earlier is an additional effect. Finally, modulation of estrogen biosynthesis and estrogen signaling pathways which enhance cellular proliferation (103), constitutes another important function of melatonin, of particular relevance to breast cancer, as discussed earlier.

1.7.1.5 INDUCING ANGIOGENESIS

Tumor-induced angiogenesis is important for the sustained supply of cancer cells with oxygen and nutrients. It also provides the route for metastatic dissemination. As the tumor grows more than a few millimeters in diameter, hypoxia leads to the production of factors that promote angiogenesis, the most important of them being vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), angiogenin and epidermal growth factor (EGF). The anti-angiogenic capacity of melatonin has been demonstrated in both in vitro studies of breast cancer cell lines and in vivo models of breast cancer (127-129).

1.7.1.6 RESISTING CELL DEATH

Apoptosis, or programmed cell death, is a process for removing cells that are no longer needed or are damaged beyond repair, in an ordered way, without disturbing their neighboring cells. Once activated, it proceeds through a series of specifically ordered set of steps, ending in the neat packaging of the cellular contents and their opsonization for phagocytosis by tissue macrophages. The process is safeguarded by a set of proteins called caspases. Activation of caspases-8 and -9 sets the process in motion. Cancer cells have developed resistance to this mechanism, which constitutes a characteristic of possibly all types of cancers (113). The addition of melatonin to MCF-7 cultures increases apoptosis by activating caspase-7 (130).

1.7.1.7 AVOIDING IMMUNE DESTRUCTION

The theory of immune surveillance advocates that the immune system surveils the organism, detecting and destroying any emerging cancer cells. As a consequence, tumors that develop are the result of defective immune surveillance. The theory seems to be supported by an increased frequency of certain types of cancers in immunocompromised individuals, particularly those that are virus-associated (131). Furthermore, the recent advances in immunotherapy for cancer treatment provide an additional therapeutic modality against tumors with reduced immunogenicity (132). Melatonin has been shown to exert immunomodulatory effects both in vitro and in vivo (133, 134). High concentrations of melatonin have been found in the bone marrow of rats, even when their pineal has been removed, indicating local production (135). In animal experiments, the addition of melatonin to the diet led to an increased number of Natural Killer cells (136). In humans, melatonin administration in cancer patients, increased the number of T helper ($CD4^+$) lymphocytes (136).

1.7.1.8 DEREGULATION OF CELLULAR ENERGETICS

Cancer cells exhibit increased metabolic demands to maintain their high proliferating rate. In 1924, Otto Warburg observed that malignant cells preferentially convert glucose to lactate, relying on glycolysis rather than oxidative phosphorylation for their energy production, even under conditions of adequate oxygen availability (137). This phenomenon is called “aerobic glycolysis”, or the “Warburg effect” and it also occurs in normal proliferating cells. Aerobic glycolysis produces significantly fewer ATP molecules than oxidative phosphorylation and this justifies the high glucose uptake of the tumors. However, it does raise questions regarding the advantage of such a metabolic shift. Warburg attributed this phenomenon to mitochondrial malfunction (137) and there is research suggesting that in some tumors the transport of pyruvate inside the mitochondria is indeed impaired. Melatonin has been shown to enhance mitochondrial pyruvate uptake in various solid tumors (138). In the case of breast cancer, it attenuates the extent of the Warburg effect during nighttime hours (139). Moreover, it is involved in the regulation of mitochondrial homeostasis (140).

1.7.1.9 UNLOCKING PHENOTYPIC PLASTICITY

Phenotypic plasticity refers to the differential expression of the genotype, depending on environmental variations. In the case of cancer, it involves the alternation between the epithelial and mesenchymal states and the expression of different traits that contribute to tumor heterogeneity, ultimately promoting

tumor progression, metastatic spread and even therapeutic resistance. Epigenetic modification is involved in the process, switching on or silencing the respective genes. There is evidence of an alteration in the tumor metabolic phenotype from Warburg-type to oxidative phosphorylation, under the influence of endogenously produced and exogenously administered melatonin (141). This, together with the role of melatonin in epigenetic changes could provide insights on novel therapeutic strategies.

1.7.2 ENABLING CHARACTERISTICS

1.7.2.1 TUMOR-PROMOTING INFLAMMATION

Inflammation is universally present in every neoplastic lesion, representing an effort of the immune system to eliminate the tumor. Nevertheless, this innate response of the organism also entails an unexpected effect: local inflammation can contribute to tumorigenesis by the release in the tumor microenvironment of substances that promote cellular proliferation, EMT, angiogenesis and ultimately invasion and metastasis (114). Furthermore, the production by inflammatory cells of reactive oxygen species, with its high mutagenic capacity, can further accentuate the malignant transformation. Melatonin's ancestral role as an antioxidant and free radical scavenger is pivotal in mediating its anti-inflammatory role in the tumor microenvironment (142).

1.7.2.2 GENOME INSTABILITY AND MUTATION

Progression to neoplasia entails the accumulation of genetic alterations in the cellular genome. As a consequence, certain mutations confer a selective advantage allowing for the growth of a subclone and its preeminence in the tumor. Genome instability is manifested in the majority of the tumors and represents a cardinal sign of the process of carcinogenesis and a necessary condition for the acquisition of the hallmarks (114). Melatonin preserves genomic stability via its prime function as an antioxidant and free radical scavenger, reducing the likelihood of DNA damage (143). Likewise, it exerts a positive effect on DNA repair pathways.

1.7.2.3 NON-MUTATIONAL EPIGENETIC PROGRAMMING

The emerging field of epigenetics has provided significant insights into how environmental factors can modify gene expression without altering the underlying DNA sequence. Gene expression can be altered through direct DNA methylation or histone acetylation, conferring diverse properties on the neoplastic cells. Inside the tumor, epigenetic programming is driven, in part, by hypoxia and limited nutrient availability. These modifications contribute to

tumor heterogeneity and enhance phenotypic plasticity, thereby potentially promoting further the malignant progression (115). Melatonin has been found to play a role in DNA methylation, primarily through its effect on DNA methyltransferases.

1.7.2.4 POLYMORPHIC MICROBIOMES

A diverse population of bacteria, viruses and fungi, collectively referred to as the microbiome, colonize the skin and mucosa of the human body, particularly within the gastrointestinal tract, but also the respiratory and urogenital tracts and the breast. A growing body of evidence indicates that the microbiome plays a significant role in modulating health and disease (115). Several pathologic states have been linked to the disruption of the gut microbiome, also called dysbiosis (144). The gut microbiome produces melatonin locally, which helps promote the integrity of the intestinal barrier, but also possibly affects the central production of melatonin by the pineal. This is accomplished by the production of serotonin in the intestinal lumen and the regulation of AANAT, which catalyzes the rate-limiting step in melatonin biosynthesis (145). Melatonin has been shown to modify the bacterial species constitution and decrease their translocation by maintaining the intestinal barrier (144). A study of 76 women with hormone receptor positive breast cancer, revealed altered microbiome composition and metabolic activity, along with gut dysbiosis and decreased melatonin serum levels, compared to healthy controls (146). However, the role of the gut microbiome in health and disease, and its potential therapeutic implications remains to be elucidated. What is more, bacteria have been observed in breast cancer cells in in situ tumors and there is growing research evidence that they can enhance cancer progression in the tumor microenvironment (100)

1.7.2.5 INNERVATION

Innervation is increasingly being recognized as a basic component of the tumor microenvironment. Tumors can actively promote axonogenesis by secreting neurotrophic factors such as nerve growth factor (147). Autonomic, somatosensory and CNS neurons promote tumor progression by enhancing proliferative signaling, resistance to apoptosis, immune evasion and metastasis, through neurotransmitters signaling to receptors on cancer and stromal cells (116). In breast cancer, the identification of nerve fibers, especially in the front of the tumor, is associated with higher histological grade, lymph node metastasis and worse prognosis (148). So far, no research has been conducted investigating the effect of melatonin on neuronal development in the tumor microenvironment. However, its anti-inflammatory and

immunomodulatory effects can counteract innervation-induced cancer progression in the tumor bed (129).

1.8 MELATONIN AND BREAST CANCER

1.8.1 THE "MELATONIN HYPOTHESES"

From its initial discovery and over the years, several theories have been proposed to explain the role of the pineal gland and melatonin and their possible association with breast cancer. To date, three "Melatonin Hypotheses" have endured, having substantially shaped the research in this field.

The first hypothesis, namely that the pineal gland is a neuroendocrine transducer, was proposed by R. Wurtman and J. Axelrod in 1965 and coincided with the emergence of the field of neuroendocrinology, which aimed at establishing the connections between the nervous and the endocrine systems. According to the authors, the pineal fulfilled the criteria of such a transducer, in that it received input from nerve endings and responded to them by releasing a hormone. In other words, it was able to convert a photic signal into an endocrine output. At that time, the connection between melatonin secretion and the photoperiod were already under investigation, together with its effect on gonadal physiology (54, 149). The hypothesis has been particularly influential, providing the framework for the field of chronobiology.

In 1978, M. Cohen suggested a second hypothesis, probably the first to associate melatonin with breast cancer. By that time, it was known that melatonin had an inhibitory effect on ovarian function, and that it could affect tumor induction and progression in experimental models. It was also known that its production diminished with increasing age. Taking those things together, he proposed that reduced melatonin production, such as that observed with old age, can produce a state of "relative hyperestrogenism" and thereby promote the development of breast cancer (150). Put differently, a decline in melatonin production, could lead to reduced control over the biosynthesis of estrogens, allowing their levels to increase and, in theory, stimulate the proliferation and eventually the neoplastic transformation of the breast epithelium. Partial support for the theory came from subsequent publications reporting lower 24h urine melatonin concentrations in patients with advanced breast cancer, compared to healthy controls (151, 152), and also lower plasma melatonin concentrations in women with hormone receptor-positive breast cancer, in proportion to the degree of receptor expression (153). Nevertheless,

while the hypothesis has generated subsequent research, it remains speculative requiring further mechanistic validation on the possible causal underlying pathways.

The third hypothesis came almost a decade later and was proposed by R. Stevens in 1987, postulating reduced melatonin production due to artificial light and low frequency electric and/or magnetic radiation. Based on animal experiments, he argued that exposure to electromagnetic fields or conditions of constant illumination, act as a "functional pinealectomy" reducing melatonin production and consequently increasing susceptibility for breast cancer. Studies on electricians have in part confirmed a negative impact of electromagnetic fields on melatonin production (154-156). Animal experiments have demonstrated that melatonin suppression under prolonged/constant illumination can accelerate breast cancer progression. The theory has played an important role in promoting epidemiological research on night shift work as well as research on chronodisruption.

1.8.2 MELATONIN-ESTROGEN INTERACTIONS

Melatonin can counteract the proliferative effect of estrogen on mammary tissue through three mechanisms (Figure 8): i) suppression of steroid synthesis by the gonads, ii) suppression of ER, acting as a SERM and iii) inhibition of aromatase, acting as a SEEM. We will examine each of the three individually.

As mentioned earlier, a decrease in serum melatonin levels occurs with the onset of puberty, though the directionality of this association is unclear. A study conducted in Finland, demonstrated during the dark season, elevated melatonin secretion and reduced ovarian activity with lower estrogen concentrations (157). A regulatory effect of ovarian steroid production by melatonin has been demonstrated in human granulosa luteal cells (103). The findings point toward the attenuation of ovarian steroidogenesis by melatonin.

Melatonin can act as a SERM, much like tamoxifen. Contrary to tamoxifen, it does not bind to the ER. It appears that it reduces the expression of ER and inhibits binding of estrogen-ER complex to the ERE. This action is thought to be mediated by the activation of the MT1 receptor (103). This effect is supported by research demonstrating that transfection of MT1 receptors to both hormone receptor positive and negative breast cancer cells, potentiates the tumor-suppressive effect of melatonin (158, 159).

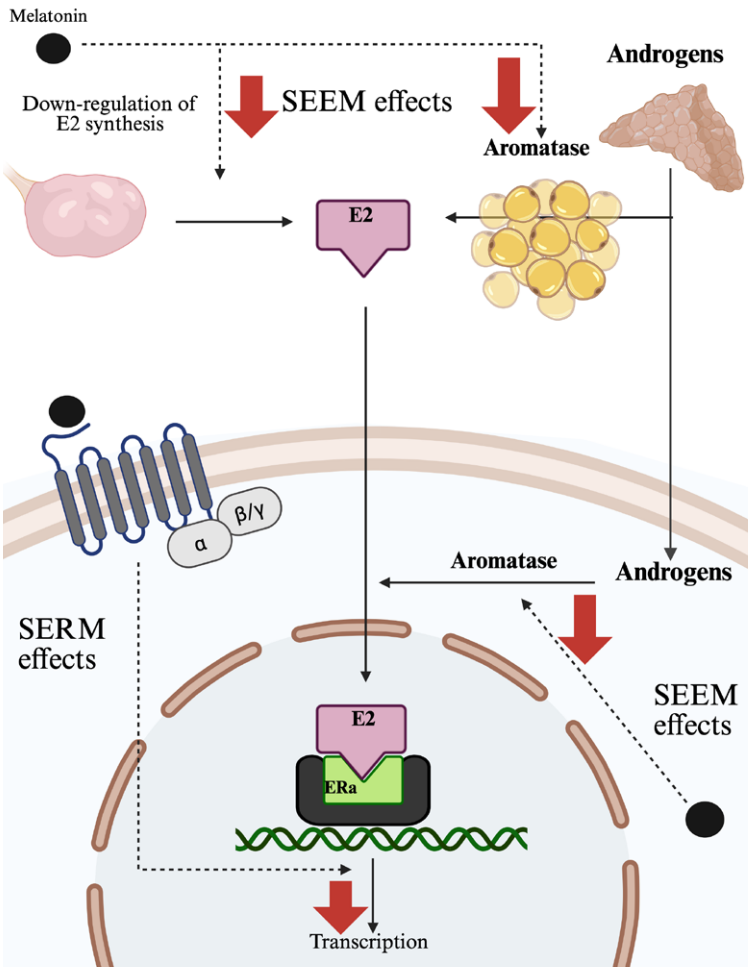

Figure 8: *Melatonin-estrogen interactions (adapted from Sanchez-Barcelo, E., (103), created with Biorender.com)*

Melatonin is perceived to act as a SEEM through its effect on aromatase, much like the aromatase inhibitors used for the treatment of breast cancer. It has been shown to decrease the synthesis but also the activity of aromatase (160-163). Along those lines, melatonin inhibits aromatase activity in the tumor bed, thereby reducing local estrogen synthesis and estrogen-mediated proliferative signaling. The anti-aromatase effect of 20 nM of melatonin was demonstrated to be as potent as 20 nM of letrozole (164).

1.8.3 EXPERIMENTAL EVIDENCE

Most in vitro studies with breast cancer cell lines have used MCF-7 cells. The cell line was isolated in 1973 from the pleural effusion of a breast cancer patient who developed metastatic disease (165). It corresponds to the Luminal, hormone receptor positive/HER2 negative breast cancer subtype. The study of that particular cell line has yielded more information than any other. An additional popular cell line is the MDA-MB-23 corresponding to triple negative breast cancer.

A number of studies have shown the inhibitory effect of melatonin on both ER positive and negative breast cancer cell lines (120, 166, 167). Its ability to promote apoptosis has been documented in both ER positive and negative breast cancer cells (130, 168-170). It has also been shown to affect the stability of the telomeres. In ER positive breast cancer, it suppresses estrogen-induced activity of telomerase (126). Furthermore, it reduces tissue invasion and metastasis (124, 171, 172). It affects epigenetic changes by promoting DNA methylation and exerting anticancer effects (173).

In interpreting the findings, it should be kept in mind that many of the effects that have been described in preclinical studies, were achieved in culture media where the concentration of melatonin was in the μM to mM range, much higher than the physiological nM (174).

Multiple studies have been conducted using animal models and controlling circulating melatonin levels via melatonin administration, alterations in the light/dark cycle or surgical interventions, such as pinealectomy. Long-term supplementation of melatonin appears to reduce the incidence of spontaneous breast cancers in mice (76, 175). Administration of melatonin increases the latency period and decreases tumor size and metastases (176). It also inhibits tumor growth (128) and reduces the effect of bisphosphenol (synthetic estrogen) on tumor proliferation (177). Furthermore, melatonin was found to inhibit the development of DMBA-induced mammary tumors, whereas pinealectomy enhanced their development (178). The effect of LAN on tumor growth rate was successfully counteracted with the supplementation of melatonin, via global DNA methylation (179).

1.8.4 EPIDEMIOLOGICAL EVIDENCE

Chronodisruption due to night shift work has been investigated under the light of cancer incidence and risk. A pioneer in that was the Nurses' Health Study, investigating the incidence of breast cancer in nurses working on night shifts (180). It included 78,652 women and had a follow-up period of ten years. Information was also obtained concerning night shift work before study enrollment. Findings indicated a moderate increase in the risk of breast cancer when working on night shifts 1-14 years or 15-29 years. The risk was further significantly increased when working 30 or more years. In a more recent publication, compiling data from the Nurses' Health Study (1988-2012; n=78,516) and the Nurses' Health Study II (1989-2013; n=114,559), the authors concluded that breast cancer risk increased with long-term night shift work, especially when done during young adulthood (181).

Two studies comparing night shifters to day workers, found a higher risk for breast cancer in women who started working at night before their first full-term pregnancy, compared to those who started night shifts afterwards (182, 183). The finding is explainable knowing that breast tissue attains its final maturation only after full-term pregnancy and breastfeeding, highlighting the importance of age at onset of night shift work.

In light of existing evidence, the International Agency for Research on Cancer, concluded in 2007, that "shift work that involves circadian disruption is probably carcinogenic to humans" and categorized it as a Group 2A ("probable") carcinogen (184). Nevertheless, the committee recognized the limitations of the epidemiological studies, particularly concerning exposure, their retrospective design and the existence of confounding factors, and suggested further research with more detailed information concerning the patterns of night shift. Subsequent studies showed variable and often conflicting results (185). The most recent systematic review and meta-analysis (SR/MA) including 26 studies, found a significant slightly increased risk for breast cancer in short-term, but not in long-term shift workers (186). A study of the epidemiologic literature from 1996 to the present time found that results support the existence of an association between night shift work and breast cancer risk, which is subject to duration, and patterns of rotation, as well as genetic susceptibility (42).

Visual impairment, and more specifically blindness, has been associated with undisturbed melatonin production due to the presumed lack of light perception. This conjecture has prompted epidemiological research investigating the

incidence of breast and other types of cancers in individuals with blindness and visual impairment (187-194). The findings of the studies are inconsistent, but one has to keep in mind that there are a lot of confounding factors involved in the interpretation of the results. Concerning melatonin in particular, ophthalmological studies have shown that even in individuals with total blindness, exposure to bright light can suppress melatonin production, suggesting that photic signals can, at least in some, be transmitted from the retina to the hypothalamus (195-197). Furthermore, accumulating evidence indicates substantial interindividual variability in the degree of melatonin suppression by LAN (198-200). At present the precise impact of visual impairment on melatonin production remains unclear.

2 AIM

The aim of this thesis was to elucidate the role of melatonin in breast cancer. In order to attain this, we examined the topic from several distinct perspectives.

The specific aims of the individual studies included:

1. To examine the association between melatonin intake and survival outcomes (OS, BCSS) in post-menopausal patients diagnosed with early breast cancer, in order to explore the possibility of a protective role of melatonin intake on the survival of those patients.

2. To investigate the expression of MT1 melatonin receptor expression in ER positive, HER2 negative, invasive ductal breast carcinoma in post-menopausal women, and its relationship with known prognostic and predictive factors (ER, PR, Ki67, p53, tumor size, tumor grade, patient age, lymph node status, and NPI), OS and BCSS.

3. To assess cancer risk overall and for different cancer types in visually impaired individuals, employing visual impairment as a potential proxy for undisturbed melatonin production, unaffected by LAN.

4. To investigate the effect of melatonin addition to systemic oncologic treatment in patients with stage IV breast cancer, in order to address the potential utility of melatonin as an adjunct to systemic oncologic treatment in that particular group of patients.

3 PATIENTS AND METHODS

Table 1. *Characteristics of included studies*

	Paper I	Paper II	Paper III	Paper IV
Study design	Retrospective registry-based cohort study	Retrospective cohort study	Retrospective registry-based cohort study	Systematic review and meta-analysis
Inclusion criteria	Female patients ≥ 55y operated for early BC from Jan.1, 2005 to Dec. 31, 2015	Patients operated for BC at Sahlgrenska U.H. from Jan.1, 2003 to Dec. 31, 2004	Men and women ≥ 40y diagnosed with varying degrees of visual impairment	RCT of patients with stage IV BC, on systemic therapy with or without melatonin
Exclusion criteria	Prior/bilateral BC, stage IV BC, neo-adjuvant treatment	Neo-adjuvant treatment, bilateral BC	Previous diagnosis of visual impairment in the control group	Animal / preclinical studies, reviews, non-English studies
Country	Sweden	Sweden	Sweden	Italy
Patients included	37,075	118	290,958	124
Diagnosis	Early BC	Early BC	Various types of cancers†	Stage IV BC
Intervention	Melatonin tablet ingestion	IHC staining for MT1	---	Melatonin
Control	No melatonin intake	---	Population controls without visual impairment	Systemic oncologic treatment
Outcomes	5-year and 10-year BCSS and OS	MT1 assessment, correlation with ER, PR, Ki67, p53, tumor size, NHG, patient age, nodal status, NPI, OS, BCSS.	Incidence of cancer, overall, and of various types of cancers†	Tumor response, 1-year survival
Follow-up	Sep. 1, 2016	Dec. 1, 2023	Dec. 31, 2021	---
Ethical approval	Yes	Yes	Yes	Not required

† Oral neoplasms, esophagus, stomach, small intestine, colon, rectum, liver, pancreas, lung, melanoma, skin, breast, uterine, ovary, prostate, bladder, brain, thyroid, lymphoma/leukemia, metastasis.

BC: breast cancer, IHC: immunohistochemical, RCT: randomized controlled trials

3.1 PAPER I

3.1.1 STUDY DESIGN

A retrospective registry-based study compiling data from the Swedish Breast Cancer Registry, the Swedish Prescribed Drug Registry and the Swedish Cause of Death Registry. Data from the registries were combined to create a cohort of patients to determine the effect of melatonin ingestion in overall survival (OS) and breast cancer-specific survival (BCSS) of patients diagnosed and treated for early breast cancer.

3.1.2 PATIENT SELECTION AND INCLUSION

Female patients aged 55 years and older (postmenopausal) diagnosed and treated for primary breast cancer between January1, 2005 and December 31, 2015. Patients with a breast cancer diagnosis prior to January 1, 2005, were excluded. Similar for patients with stage IV at diagnosis and for those who received neo-adjuvant therapy.

3.1.3 DATA COLLECTION

Data were retrieved from the corresponding registries and included patient age at diagnosis, method of diagnosis (screening or not); information on tumor biology: tumor size, Nottingham histologic grade (NHG), ER, PR, Ki67, HER2 status; type of breast and axillary node surgery and type of adjuvant treatment (chemotherapy, radiation therapy, endocrine treatment and anti-HER2 therapy).

A total of 38,120 patients were initially identified and after application of the exclusion criteria, 37,075 remained. Of those patients, 926 were found to have been prescribed melatonin, consisting 2.5% of the cohort. Overall, there were no major differences between the melatonin and non-melatonin groups concerning patient and tumor characteristics.

3.1.4 OUTCOME MEASURES

Endpoints included BCSS and OS at 5- and 10-years post-diagnosis.

3.2 PAPER II

3.2.1 STUDY DESIGN

Retrospective cohort study utilizing tissue samples from patients operated for breast cancer at Sahlgrenska University Hospital, using immunohistochemistry (IHC) to assess the expression of MT1 melatonin receptors in breast cancer tissue and axillary metastases, and investigate its relationship with patient and tumor characteristics, axillary metastasis, patient survival and recurrence-free interval (RFI).

3.2.2 PATIENT SELECTION AND INCLUSION

Female, postmenopausal patients operated for primary ER positive, PR positive/negative, HER2 negative, invasive ductal breast carcinoma, at Sahlgrenska University Hospital between January 1, 2003, and December 31, 2004. Patients receiving neo-adjuvant therapy and patients with bilateral breast cancer were excluded.

3.2.3 DATA COLLECTION

A combined number of 153 patients were identified according to inclusion and exclusion criteria. Further analysis of the cohort led to the exclusion of three patients due to bilateral breast cancer and inability to retrieve paraffin blocks led to a total of 118 specimens for immunohistochemical staining. Melatonin antibodies were utilized to detect melatonin receptor expression, which was subsequently evaluated both as percentage of stained cells and staining intensity (1: faint, 2: weak, 3: moderate, 4: strong). The weighted index (WI) was calculated by multiplying the two. Follow-up for survival and recurrence calculation was until December 1, 2023.

3.2.4 OUTCOME MEASURES

Both percentage staining and WI were correlated with patient (age), and tumor characteristics (size and grade, ER, PR, Ki67, p53), lymph node status and the calculated Nottingham Prognostic Index (NPI). Univariate and multivariate Cox regression for OS, BCSS and RFI utilized the same parameters plus MT1 percentage and WI.

3.3 PAPER III

3.3.1 STUDY DESIGN

Retrospective registry cohort study combining data from the National Patient Registry of Sweden, Statistics Sweden (socioeconomic data) and Cause of Death Registry, to investigate the risk of various types of cancer among individuals with visual impairment, each matched for age, sex and county of residence, with 5 population controls.

3.3.2 PATIENT SELECTION AND INCLUSION

Adult Swedish men and women aged 40 years and older, with H54 diagnosis, i.e. diagnosed with various degrees of visual impairment (from mild visual impairment to total blindness) between 2002 and 2018, each matched by age, sex and region of residence, with 5 seeing controls. Any prior H54 diagnosis in the controls served as exclusion criterion.

3.3.3 DATA COLLECTION

Data were retrieved from the corresponding registries. In total, 48,493 individuals with visual impairment were identified, together with their 242,465 controls. Between the two groups of the cohort, the mean Charlson Comorbidity Index (CCI) was significantly higher for individuals with visual impairment. Furthermore, cancer prevalence during the last 5 years was also significantly higher in the same group. Follow-up was until December 31, 2021.

3.3.4 OUTCOME MEASURES

Primary outcome was overall cancer incidence. Secondary outcomes comprised the incidence of specific cancer types (oral neoplasms, esophagus, stomach, small intestine, colon, rectum, liver, pancreas, lung, melanoma, skin, breast, uterus, ovary, urinary bladder, brain, thyroid, lymphoma/leukemia, solid metastasis).

3.4 PAPER IV

3.4.1 STUDY DESIGN

Systematic review and meta-analysis investigating the effect of adjunct melatonin supplementation to systemic oncologic treatment, in tumor response and survival of patients with stage IV breast cancer. Prior to its commencement, the study was registered in the International Prospective Register of Systematic Reviews (PROSPERO) database with registration number CRD42025644224.

3.4.2 STUDY SELECTION AND INCLUSION

A systematic search of the literature was conducted in four databases: PubMed, Embase, Scopus and Cochrane CENTRAL. The search algorithm was created using Medical Subject Headings (MeSH), key words and Boolean operators. Studies were initially evaluated by title, by two independent reviewers and disagreements were solved by a third. Inclusion criteria involved: randomized controlled trial, patients with stage IV breast cancer being administered any kind of systemic oncologic therapy (chemotherapy, immunotherapy, hormonal therapy) and melatonin administration in the experimental arm. The study should also contain information on tumor response and survival. Pre-clinical and animal studies, previous meta-analyses and non-English studies were excluded. A manual search was also performed from citations of related articles. Eligible articles were then screened by abstract, and text and three studies were finally selected, with a total of 124 patients.

3.4.3 DATA COLLECTION

Data on tumor response and 1-year survival were independently extracted by two reviewers. No discrepancies were noted. Risk of bias assessment and quality of studies were also conducted by two independent reviewers and disagreements were resolved through discussion.

3.4.4 OUTCOME MEASURES

The endpoints of interest were tumor response and 1-year survival. The odds ratio (OR) was calculated for both parameters. Additional calculated parameters included heterogeneity assessment of included studies, percentage of agreement and Cohen’s kappa to evaluate inter-reviewer agreement, absolute risk reduction (ARR) and number needed to treat (NNT).

4 STATISTICAL ANALYSES

4.1 PAPER I

Standardized statistics were used for descriptive data. Continuous data were presented as median and interquartile range. Categorical variables were presented as number and percentages. Univariable and multivariable Cox regression analyses were performed. Parameters in the multivariable analysis for both BCSS and OS, included: patient age, tumor size and grade, Ki67, ER and HER2 status, axillary lymph node status and exposure/ no exposure to melatonin. The results were presented in tables and visualized in Kaplan-Meier curves and Cox regression analyses graphs. Analyses were performed using SPSS version 28 (Chicago, IL, USA).

4.2 PAPER II

Normal distribution of the variables was assessed with the Shapiro–Wilk test before calculating Pearson's correlation coefficient. Differential expression of MT1 expression and WI according to grade was evaluated using the Kruskal–Wallis test and according to lymph node metastasis, using the Mann–Whitney U test. Cox regression was used for survival analysis. The results were presented in tables and figures. The NPI was calculated according to the standard formula:

$$\mathrm{N}PI{=}LN\ (0{=}1,\ 1{-}3{=}2,{>}3{=}3) + Grade{+}\ 0.2{\times}tumor\ size\ (\mathrm{cm})$$

All statistical analyses were performed using the SPSS 29.0.1.1 statistical package (IBM Corp., Armonk, NY, USA).

4.3 PAPER III

Descriptive statistics were presented as counts with percentage (categorical variables) or medians with standard deviations (SD) (continuous variables). Event rates were computed as number of events per 1000 person-years, with 95% Poisson confidence intervals. For all outcomes, Cox regression analysis was performed, with adjustment for age, sex, baseline year and previous cancer, the latter depending on the outcome examined. The findings were presented in tables and figures. Analyses were performed using R version 4.2.2 and R-studio version 2023.03.0+386.

4.4 PAPER IV

The OR for tumor response and 1-year survival and the study heterogeneity were calculated using RevMan 5.4. The fixed effects model was employed, using the Peto correction to account for the small number of studies and included patients. Inter-reviewer agreement as percentage and Cohen's kappa, ARR and NNT, were calculated using SPSS version 29.0.2.0. (Chicago, IL, USA). The results were reported in tables, figures and forest plots. The small number of included studies did not allow for the creation of funnel plots.

5 METHODOLOGICAL CONSIDERATIONS

The first recorded randomized clinical trial in the history of medicine, was effected in Sweden, and was planned by King Gustav III himself (201). At that time, coffee consumption had been banned in Sweden, because of the presumed association between coffee drinking and delinquent behavior. In the year 1771, the King commissioned a study enrolling a pair of identical twins as its subjects. The twins had been sentenced to death and were promised that their sentences would be commuted to life imprisonment if they agreed to participate. After their "voluntary informed consent", one of them was assigned to drink three pots of coffee every day, and the other, three pots of tea, for the remainder of their lives. The twin participants outlived both doctors supervising the trial, as well as the King. At the age of 83, the tea drinker died, and the coffee-drinker won. The ban was raised during the 1820's and today, everybody in Sweden is free to enjoy the beloved fika!

Since then, research methodology, and of course informed consent, enrollment and randomization have improved considerably. Methodology signifies a cardinal feature in any study design and constitutes a separate section in the published articles. It is important for the minimization of bias and for reproducibility. It can "make or break" a study and become a major issue of critique from reviewers. But, above all, if not planned and implemented correctly, it can lead to erroneous results and false conclusions.

The foundations of modern medicine go back to classical Greece, where Hippocrates (460-370 BC.), considered as the "father of medicine", postulated that the practice of medicine should be based on observations, reasoning and experience of the physician, applied according to his/her "ability and judgement" (202, 203). However, even 2500 years ago, Hippocrates realized that both ability and judgement could go wrong (202). Fast forward to the 1970's when the necessity of bolstering the empirical practice of medicine started to be articulated through the voices of D. Sackett, A. Cochrane, and D. Eddy, who proposed the employment of evidence-based rules for directing clinical decisions (204). In 1991, G. Guyatt coined the term "evidence-based medicine" (EBM) introducing a new tool with the intention of using research-generated evidence to improve patient care (205, 206). And so begun the era of EBM, which originally focused on the critical appraisal of the published research, the advance of systematic reviews and the development of guidelines

for clinical practice. It maintained that medical practice should be grounded on the strongest available evidence, and that truth is best approached through comprehensive, rather than selective consideration of the evidence (204).

Soon however, came the realization, that not all evidence is created equal. The iconic Hierarchy of Evidence pyramid was designed, under the consideration that the higher the quality of the evidence, the closer the estimates are to the truth. The original from 1991 (Figure 9) contained eight levels, with a focus on trial design. RCTs were placed at the apex of the pyramid, representing the most rigorous form of trial design. At the very bottom of the pyramid, the expert's experience. Hippocrates strikes back! Through the years, multiple versions of the pyramid have been proposed, and one could not help but speculate if they represent, to some degree, a disagreement among scientists of what the "best evidence" really is. After all, a "better" method is not bound to produce more reliable evidence. An inadequately designed randomized controlled trial could produce inferior evidence compared to a well-designed and conducted observational study.

Figure 9. *Hierarchy of evidence pyramid (adapted from Djulbegovic, B., (204), created with Biorender.com)*

A point worth mentioning, for the sake of clarity, is that in multiple versions of the pyramid, the apex is occupied by SR/MA. This is however considered misleading by Guyatt himself, since SR/MA is an approach to evidence synthesis and not a study design per se (204). At the end of the day, the quality of a SR/MA is just as good as the quality of the studies it includes. In order to evaluate that, a more sophisticated tool termed Grades of Recommendation, Assessment, Development and Evaluation (GRADE) was introduced in 2004. It addresses not only study design, but also, among other factors, the risk of bias, the variability of the results among included studies and the magnitude of the effects measured.

Reporting the findings has also come to be regulated by widely accepted international guidelines, which aim at improving the reliability and validity of the published medical literature by fostering transparent, thorough, and precise reporting standards. The EQUATOR network represents such an example, with 695 different guidelines, depending on study type. Adhering to these guidelines enhances the quality of data reporting and provides authors with a structured framework on the way it should be conducted.

5.1 CRITICAL APPRAISAL OF THE PAPERS

With the aim of putting the knowledge gained so far into practice, we tried to do a critical appraisal of the thesis papers, based on a set of questions compiled from du Prel J.B et al (207) and the Critical appraisal Checklist for Cohort Studies from the Johanna Briggs Institute. The checklist is by no means extensive, standardized or applicable to all diverse types of research included in this thesis. It represents nonetheless, an attempt to look back at the investigations carried out and assess, as objectively as possible, what was done well and what was not. The results are presented in Table 2.

5.2 CONSIDERATIONS IN RETROSPECTIVE REGISTRY STUDIES

Retrospective registry studies represent an important research methodology which allows the investigation of a disease, treatment or subgroup of the population, by utilizing real-world data. This confers external validity. In addition, they allow room for long-term effects and provide large numbers for the calculation of statistical results. Nevertheless, they can be subject to certain limitations if care is not taken in their design and interpretation.

Table 2: *Critical appraisal of the Papers*

	Paper I	Paper II	Paper III	Paper IV
Design and Methods				
Is the aim of the study clearly stated?	⊕	⊕	⊕	⊕
Are the study population and the inclusion/exclusion criteria clearly stated?	⊕	⊕	⊕	⊕
Is it clear why the particular study design was chosen?	?	⊕	⊕	⊕
Are the methods of measurement appropriate for the target variables?	⊕	⊕	⊕	⊕
Is there information regarding missing values/loss to follow-up?	?	⊖	⊖	NA
Is the follow-up time complete?	⊕	⊕	⊕	NA
Is the follow-up time sufficient for the effect in question to occur?	?	⊕	?	NA
Analysis and Evaluation				
Are treatment and control groups similar in relation to major characteristics?	⊕	NA	⊕	NA
Are the statistical parameters selected, correct?	⊕	⊕	⊕	NA
Are the statistical analyses clearly described?	⊕	⊕	⊕	⊕
Are the statistical parameters presented appropriately, comprehensively and clearly?	⊕	⊕	⊕	NA
Are effect sizes and CI, for the principal findings, clearly reported and presented?	⊕	⊕	⊕	⊕
Are confounding factors/potential biases identified and analyzed?	?	?	⊕	⊕
Are the conclusions supported by the findings?	⊕	⊕	⊕	⊕

NA: Not applicable

Registries have traditionally been established for administrative, clinical or quality-improvement reasons, rather than research alone. As a consequence, the study population might differ from the target population of the study. This holds especially true if the registry is voluntary or influenced by factors such as disease severity or institutional practices. Care should therefore be taken for the clear definition of inclusion criteria of the study in order to ensure internal validity and avoid selection bias. A common concern is that of missing or

erroneously inserted data. Those issues seem to appear quite often in criticisms of those studies, and both can lead to reporting bias. In the case of misclassification, the researchers are often unaware. The question arises however, regarding the extent to which those data can influence the outcome measured.

An additional concern is that of time, especially in cases of multiple factors and/or multiple outcomes under investigation. Since registries accumulate data over long periods of time, multiple diagnoses, treatments and factors may be introduced through the years. When it is important to know which factor or outcome came first, the problem can be solved by conducting a lag-time sensitivity analysis.

A major potential limitation in registry-based studies is confounding. This occurs then an independent condition/factor can have an effect on both exposure and outcome, thus leading to a distorted association between them. Closely related is confounding by indication, in which a parameter used as an indication of the exposure is responsible for both exposure and outcome. The best way to avoid confounding is through randomization, but this is not feasible in cohort studies. Alternatively, the use of matching or propensity scores can be employed.

Papers I and III are registry-based, retrospective cohort studies, drawing data from National Swedish Registries. These registries have nationwide coverage and data input is done by personnel involved in healthcare. Furthermore, the personal identification number used in Sweden, allows for cross-reference between the different registries and the assimilation of data. While the possibility of missing data and miscoding cannot be excluded, available evidence suggests that the associated risk is low (208). Prior reviews suggests that the Swedish Patient Registry maintains good validity and contains relatively little missing data (208, 209).

5.3 CONSIDERATIONS IN IMMUNOHISTOCHEMISTRY STUDIES

Immunohistochemistry (IHC) is a widely used technique which utilizes specific antibodies for the detection of proteins in tissue samples. It has been widely used in everyday laboratory practice for more than half a century and has earned its place as both a valuable research tool and a complementary

method for cancer diagnosis and subtyping. As is the case with every technique, ICH is also subject to bias, which is divided into reaction bias and interpretation bias (210).

Reaction bias refers to pre-analytical factors concerned with tissue handling, processing and fixation. Ischemia time, fixation and tissue processing can significantly impact antigen preservation and can lead to epitope masking, necessitating retrieval procedures. The latter two can affect staining intensity (211). Interpretation bias refers to analytical factors such as the choice of antibody, staining protocols and interpretation and scoring. The choice of the antibody applied is of importance, since monoclonal antibodies have high specificity and improved reproducibility, whereas polyclonal antibodies have a higher sensitivity. On the other hand, they are subject to lot-to-lot variability, which can affect staining patterns and signal intensity, and inferior reproducibility (210). Staining procedures, such as antibody dilution and incubation time can also affect the outcome. The interpretation of the results is assessor-dependent and relies on the experience of the pathologist. The human factor in interpreting IHC may introduce biases including sampling bias, as the pathologist might select the "most representative" area of the tissue section for the assessment; inconsistencies in tissue scoring for both quantitative and semi-quantitative approaches and reporting bias in cases of lack of blinding.

During the assessment of MT1 in the study presented in Paper II, we tried to avoid those pitfalls as much as possible. The paraffin blocks were retrieved from archive of the Department of Clinical Pathology, Sahlgrenska University Hospital. For the way the tissue samples were originally handled, processed and embedded, we had absolutely no control, but slide preparation for MT1 assessment was conducted according to current standard laboratory protocol, as reported in the Paper. Several antibodies were tested before selecting the one that was finally used. The definitive choice relied on the antibody working with both positive and negative controls. Positive and negative control were done by using human pineal gland tissue. The antibody that was finally chosen is a polyclonal rabbit antibody. Staining was done using an auto stainer platform. The results were assessed by the same qualified pathologist, sub-specialized in breast pathology. Assessment of the histologic slides by a sole pathologist may represent a source of limitation, considering that IHC for MT1 does not constitute part of routine pathologic assessment and experience is therefore limited.

5.4 CONSIDERATIONS IN SYSTEMATIC REVIEWS AND META-ANALYSES

Systematic reviews and meta-analyses represent the highest methodological form for the synthesis of evidence from already conducted research. They adhere to strict methodology to be able to increase their statistical power of the results through quantitative assimilation. In order for this to be achieved, there are certain things that need to be done even before the literature is searched to ensure the credibility of the review and the confidence in its results.

The first and foremost refers to framing a clear question which the SR/MA wishes to answer. This should preferably be formulated according to the Patients, Interventions, Comparisons, and Outcomes (PICO) model and entails a decision on how broad or narrow the question is going to be. With a clearly defined research question, the eligibility criteria of the included studies can be chosen with relative ease. The study protocol needs to be clearly delineated, including the question of the study, the rationale, inclusion and exclusion criteria, the study selection process and databases that will be searched as well as the study selection and data extraction processes. The methods for assessing the risk of bias need to be decided beforehand. The same holds for data synthesis. The protocol should be registered in PROSPERO enhancing methodological transparency and preventing selective reporting by defining methods a priori to study identification and data extraction. A break in the methodology can jeopardize the credibility of the study.

The search strategy (databases used and manual search) should be presented in a clear and reproducible way to minimize publication and retrieval bias. Inadequate database coverage, the use of restrictive terms and language limitations may result in omission of relevant studies. Additional potential sources of bias include study selection and extraction. It is important that they are conducted by two independent reviewers, and that the inclusion and exclusion criteria are straightforward. The authors should decide beforehand how the disagreements will be resolved. Study heterogeneity should be checked and reported.

As stated above, the quality of the outcomes of a SR/MA depends on the quality of the included studies. Consequently, a careful evaluation of the risk of bias in the selected studies is required. Given that such assessments may be susceptible to subjective bias, the best practice dictates it is undertaken by two

independent assessors. Depending on the quality of the study, there is a variety of widely accepted tools that can be used e.g. Cochrane Risk of Bias tool.

An additional potential risk for the credibility of a SR/MA is that of publication bias. By this, we mean that studies that favor a particular outcome are submitted and published, as opposed to studies containing negative or unfavorable outcomes. This can directly affect the outcome. Another potential source of bias, closely related to publication bias, is the small study effects. This refers to the observed tendency of smaller studies to report larger effects. This can pose a threat to the validity of the SR/MA, especially if it predominantly relies on small studies. Funnel plots can provide indirect evidence of such biases, but their use is limited when the number of included studies is small.

Finally, consideration should be given to the certainty of the evidence and its generalizability. After all, statistical significance does not necessarily guarantee clinical relevance or significance. Tools such as GRADE can assist in evaluating the overall certainty of evidence, checking for aspects such as study quality and applicability.

In the SR/MA in Paper IV, we tried to follow the methodology as outlined in the Cochrane Handbook (212) in order to ensure the credibility of the review and meta-analysis. The studies retrieved were only three, despite extensive literature review, combining both structured search of four databases and thorough manual search of related literature. Our findings are therefore subject to criticism, in spite of the large effect sizes. Nevertheless, all efforts were made to ensure methodological integrity in aspects within our control.

5.5 CONCLUDING REMARKS

In his multi-cited article from 2005, J.Ioannidis argues “Why most research findings are false” (213). He debates that the probability that a statistically significant result represents in fact a true effect, depends on i) the pre-study probability that the hypothesis is true, ii) the statistical power of the study and iii) the level of statistical significance. He broadly defines bias as factors in the design, analysis and presentation that tend to produce outcomes that should not be produced. This should not be confused however with chance variability which can cause outcomes by sheer chance, despite adequate study design and analysis. He proceeds the argument further by postulating that the results that get published may reflect, in fact, accurate measurements of the prevalent

biases. Since however, it is impossible to know with absolute certainty what the truth really is, a "gold standard" is unattainable. As a consequence, the highest-quality available evidence should come from large studies providing enhanced power, low-bias SR&Ms, better research standards and a better understanding of the pre-study odds.

In a more recent article (214), J. Ioannidis gave 12 recommendations on "How to make more published research true". Among them are the large-scale research, registration of studies and protocols, standardization of definitions and enhancement of study design. A key consideration is the reproducibility of practices and the fostering of a culture of reproducibility in the scientific community. These further underscore the importance of a detailed account of the methods section in scientific communication.

In the present thesis, we sought to produce scientific literature with as low degree of bias as possible. Of the four papers, the one that comes closer to the recommendations is Paper III, with its large sample size from a reliable source, matching of cases and controls, and robust statistical analysis, adjusting, when possible, for confounding factors such as previous cancers.

6 ETHICAL CONSIDERATIONS

Adhering to the Hippocratic doctrine “First do no harm”, the World Medical Association adopted in 1964, the Declaration of Helsinki, in an effort to provide an ethical scaffold for research involving humans. The declaration safeguards the participants’ welfare, dignity, rights and privacy, as well as the confidentiality of the information they provide. It maintains that participation necessitates an informed consent and the need for an independent committee for the ethical approval of the review protocol. It endorses the conduction of research in scientifically sound methodology, with an auspicious risk-benefit ratio and the responsibility of the scientists to communicate their results, whether positive or negative. The original text has been revised several times to adjust for current issues such as inequalities in research and genetic research. Ever since its publication, it has become a reference point for international, national and institutional research regulations.

Complementary to the Declaration of Helsinki is the Declaration of Taipei, adopted in 2006, which concerns ethical considerations on biobanks and health-related databases, beyond the provision of healthcare. Again, it stresses privacy protection of the participants and demands measures to protect potentially identifiable information and the provision of rules for data access and de-identification. The participants should be informed about the usage, storage and sharing of their data/biological materials and the right to withdraw should be provided. The abovementioned information should be included in the informed consent.

All included studies, except for the SR/MA, have been approved by the Ethical Review Board. In the SR/MA, informed consent had been obtained in all three included studies, according to the authors.

Papers I and III are retrospective registry studies and data were received anonymized, consequently, there was no risk for patient identification. Paper III used a local database for the identification of eligible patients. Once the list was compiled, the patient’s personal identification number (PIN) was used to identify the paraffin blocks containing material from the tumor and, when applicable, the axillar nodes. After that, each patient was given a serial number, and the anonymized list was sent to the pathologist. The PIN was once again used to access patient files for the retrieval of information pertaining to patient survival and recurrence.

7 RESULTS

Table 3. *Summary of findings*

Paper	Outcomes	Number of participants /Studies	Certainty of evidence (GRADE)*
I	Intake of melatonin was found to have a significant protective effect on BCSS in the univariable analysis (HR 0.736, 95% CI 0.548-0.989) which disappeared in multivariable analysis (HR 1.037, 95% CI: 0.648-1.659).	37,075	⊕⊕⊕⊖ Moderate
II	No association was found between MT1 receptor expression and patient/tumor characteristics, recurrence or survival.	118	⊕⊕⊕⊖ Moderate
III	Visually impaired had a significantly increased risk for cancer (HR 1.18, 95% CI 1.16-1.20), compared to seeing matched controls. The risk was also significantly increased for most specific types of cancers examined. The elevated risk was also found across all age-groups examined, but not between sexes. Visually impaired had a significantly increased mean CCI (1.48, SD 1.93) compared to controls (0.85, SD 1.49). When adjusted for age, sex and baseline year, risk of death in visually impaired was 60% higher (HR 1.60, 95% CI 1.58-1.63).	48,493 cases 242,465 controls	⊕⊕⊕⊕ High
IV	The addition of melatonin to oncological treatment conferred a significant advantage in tumor response: OR 2.91 (95% CI 1.29-6.57), ARR: 20%, NNT: 5.0 and in 1-year survival: OR 3.4 (95% CI 1.63-7.08), ARR: 29%, NNT: 3.4.	124 / 3[#] 117 / 2[†]	⊕⊕⊖⊖ Low

***** GRADE Working Group grades of evidence (212)
High = This research provides a very good indication of the likely effect. The likelihood that the effect will be substantially different is low.
Moderate = This research provides a good indication of the likely effect. The likelihood that the effect will be substantially different is moderate.
Low = This research provides some indication of the likely effect. However, the likelihood that it will be substantially different is high.

[#] Tumor response, [†] 1-year survival

7.1 PAPER I

The study included 37,075 female patients diagnosed with early-stage breast cancer between 2005 and 2015. Of them, 926 (2.5%) had taken melatonin during that 10-year period. There were no significant differences between the groups concerning type of surgery, tumor size and characteristics, nodal status or adjuvant treatment.

The 5-year and 10-year BCSS rates among patients who received melatonin were 97.6% and 97.0%, respectively, compared with 94.7% and 91.5% in those who did not receive melatonin ($p = 0.041$). This corresponded to a statistically significant hazard ratio (HR) of 0.736 (95% CI 0.548–0.989; $p = 0.042$). However, in the multivariable Cox regression analysis, the only variables that remained significant independent prognostic factors were age, tumor size, tumor grade, ER status, Ki67, and nodal status. Ingestion of melatonin was not found to confer any independent benefit (HR 1.037, 95% CI 0.648–1.659).

Concerning OS, the 5-year and 10-year rates were 88.9% and 74.3% in the melatonin-exposed group, compared with 86.2% and 71.0% in the non-exposed group, with an HR of 0.886 (95% CI 0.767–1.022). Multivariable Cox regression for OS identified the same independent prognostic factors as those observed for BCSS, with the addition of HER2 status.

In addition, no significant effect was found when trying to investigate the cumulative effect of melatonin on patient survival. Patients in the melatonin group were divided into low- and high-dose subgroups, with a cut-off defined daily dose (DDD) of 190, corresponding to three months of treatment. A non-significant advantage was found for the high-intake group (HR 0.525).

7.1.1 PAPER I: CERTAINTY OF EVIDENCE

The study utilizes data from three national registries investigating melatonin intake in Sweden, where melatonin was not, at that time, available over the counter, but was still a prescription drug. Taking into consideration the reliability of the Swedish registries, one can be confident about the results. The number of patients included in the study (37,075) is large and even though the percentage of them who took melatonin is small (2.5%), one can be confident that it actually is representative. An issue that has not been examined is that of the temporal aspect of melatonin ingestion in relation to cancer diagnosis.

Nevertheless, further research on the topic might yield different results and therefore the certainty of the evidence is deemed as moderate.

7.2 PAPER II

The study was conducted on tumor samples from 118 patients operated for early breast cancer at Sahlgrenska University Hospital, between January 1, 2003, and December 31, 2004. Melatonin MT1 receptor expression was assessed as both a percentage, and as WI (percentage x intensity). No significant differences in the distribution of tumor MT1 or WI were observed across the different histological grades (Kruskal–Wallis test, $p = 0.640$ and $p = 0.196$, respectively). Likewise, the median tumor MT1 and WI did not differ significantly among patients with and without axillary metastases (Mann–Whitney U test, $p = 0.776$ and $p = 0.702$, respectively).

The Spearman's correlation coefficient was used to assess the relationship between tumor MT1 receptor expression, WI, and various patient and tumor characteristics. No significant correlations were detected with patient age, tumor size, ER, PR, Ki67, p53, NHG, NPI, or MT1 expression in metastasized axillary lymph nodes. The tumor WI was evaluated in an analogous manner against the same set of variables, except that nodal MT1 expression was replaced by nodal WI. Similarly, no significant correlations were identified.

At the end of follow-up (December 1, 2023), 73 of the 118 patients (61.9%) had died, 15 of which to breast cancer (20.6%). Locoregional recurrence had occurred in 9 patients (7.7%), contralateral breast cancer in 3 patients (2.5%), and distant metastases in 17 patients (14.4%). Median overall survival was 182 months, and median BCSS was not reached.

Univariable Cox regression analyses were conducted to identify prognostic factors for OS, BCSS, and RFI. Patient age, tumor size, NHG, ER, PR, Ki67, p53, nodal status, NPI, tumor MT1 expression, and tumor WI were assessed. For OS, patient age was the only factor found to be significant (HR 1.096, 95% CI 1.067–1.126). For BCSS significant factors included tumor size (HR 1.030, 95% CI 1.004–1.056), Ki67 (HR 1.029, 95% CI 1.008–1.050), and NPI (HR 2.803, 95% CI 1.724–4.558). For RFI, significant factors included tumor size (HR 1.032, 95% CI 1.010–1.054), Ki67 (HR 1.020, 95% CI 1.000–1.039), N1 status (HR 29.767, 95% CI 3.013–294.085), N2 status (HR 24.332, 95% CI 1.412–419.289), and NPI (HR 2.450, 95% CI 1.667–3.602). In multivariable

Cox regression analyses for BCSS and RFI, with adjustment for patient age and NPI, MT1 expression and WI exhibited no significant prognostic effect.

7.2.1 PAPER II: CERTAINTY OF EVIDENCE

In this study, the use if IHC for the assessment of MT1 melatonin membrane receptors and the investigation of its relation to patient and tumor characteristics as well as NPI, BCSS, OS and RFI, constitutes an attempt to investigate if MT1 expression could represent a potential prognostic factor for postmenopausal patients with ER positive/HER2 negative invasive ductal breast carcinoma. The study included 118 patients, a sample which could be considered small, since a larger sample could potentially reveal any existing relationship. Furthermore, no significant correlation was identified with the examined clinicopathological parameters, patient survival or RFI. This is in accordance with some, but not all of the previous studies. In addition, technical issues during preparation and immunohistochemical staining, could have introduced bias, reducing the reliability of the findings. Alongside the aforementioned is that MT1 assessment is not a routinely performed in the everyday pathology practice and therefore experience with this method, as well as its evaluation, remains limited. The fact that the current and similar investigations have failed to reach a unanimous agreement, or at least a trend in the role of MT1, underscores the need for further research which may or may not yield conclusive results, deviating from the ones we presented. Subsequently, the quality of evidence of the study is deemed as moderate.

7.3 PAPER III

The study included 48,493 adults aged 40 years and over with visual impairment, and 242,465 seeing controls, matched 1:5 for age, sex and inclusion year. The median age was 77 years (IQR 64-86) and 56% of them were women. Median follow-up was 5.1 years (IQR 2.9-8.6) for the visually impaired and 6.0 years (IQR 3.6-9.8) for the controls. Age, sex, year of inclusion, previous cancers and CCI were used as baseline variables. The CCI was developed by M. Charlson in 1987 (Charlson 1987) as a tool to estimate the overall burden of comorbidities of patients, by assigning a weighted score to each of the 19 conditions included, according to its severity. The greater the score, the higher the risk for mortality and the likelihood of poor health outcomes. The CCI score is calculated by adding the weighted score of the patient's comorbidities. It is a widely used tool for the assessment of a patient's overall health burden in both clinical practice and research. The included

comorbidities as well as the scores assigned, can be seen in Table 4. Between the groups, the mean CCI was higher in the visually impaired (1.48, SD 1.93), compared to controls (0.85, SD 1.49). The 5-year prevalence of any cancer type, was significantly higher in the visually impaired group (10%), compared with the control (7.8%). When focusing on specific cancer types, prevalence rates were again higher in visually impaired.

Table 4. *Charlson Comorbidity Index*

Comorbidity	**Weighted score**
Myocardial infarct Congestive heart failure Peripheral vascular disease Cerebrovascular disease Dementia Chronic pulmonary disease Connective tissue disease Peptic ulcer disease Mild liver disease Diabetes	1
Hemiplegia Moderate or severe renal disease Diabetes with end organ damage Any tumor Leukemia Lymphoma	2
Moderate or severe liver disease	3
Metastatic solid tumor AIDS	6

During the follow-up period, 22.9% in the visually impaired group and 22.6% in the control group received a cancer diagnosis, translating to incidence rates of 43.1 (95% CI, 42.3-43.9) and 36.7 (95% CI, 36.4-37,0) per 1000 person-years, respectively and corresponding to an 18% higher risk in the visually impaired, of developing any cancer (HR 1.18, 95% CI, 1.16–1.20) after adjustment for age, sex, and baseline year. The association remained unchanged even when adjusted for prior cancer (HR, 1.17, 95% CI, 1.14–1.19). Elevated risks were observed for most site-specific cancers. Age-, sex-, and baseline year–adjusted HRs ranged from 2.59 (95% CI 2.21–3.03) for brain cancer and 1.58 (95% CI 1.23–2.05) for small intestine cancer to 1.49 (95% CI 1.30–1.72) for oral cancer and 1.19 (95% CI 1.11–1.27) for breast cancer. The estimates were unaffected further adjustment for previous cancer.

The cohort was divided into three subgroups in order to investigate potential differences among them: 40-64 years, 65-79 years and 80 years and older. Differences were observed among the subsets of the cohort. In the 40-64 subgroup, relative to sighted controls, individuals with visual impairment had a 37% higher risk of developing cancer of any type (HR 1.37, 95% CI 1.30–1.43), a finding that remained essentially unchanged after adjustment for prior cancer (HR 1.31, 95% CI 1.25–1.38). In the 65-79 subgroup, compared with sighted controls, individuals with visual impairment had a 17% higher risk of developing any cancer (HR 1.17, 95% CI 1.13–1.20), a result that remained essentially unchanged after adjustment for prior cancer (HR 1.16, 95% CI 1.13–1.20). in the last sub-set of the cohort, with individuals 80 years and older, individuals with visual impairment had a 17% higher risk of developing any cancer (HR 1.17, 95% CI 1.13–1.20) compared to seeing controls, a result that remained essentially unchanged after adjustment for prior cancer (HR 1.16, 95% CI 1.13–1.20). Statistical analysis revealed no interaction between sex and cancer of any type between the two groups.

7.3.1 PAPER III: CERTAINTY OF EVIDENCE

This retrospective cohort study also used data from Swedish national registries which, as already stated, constitute a highly reliable source. It includes a large sample of individuals with visual impairment, who are matched 1:5 to seeing controls, by age, sex and inclusion year. As a matter of fact, it constitutes the largest study conducted so far dealing with visual impairment and cancer risk. The statistical analysis is robust, and further research is not likely to provide different results. The quality of the evidence is therefore regarded as high.

7.4 PAPER IV

The study is a SR/MA examining the role of the addition of melatonin to systemic therapy in tumor response and 1-year survival, in patients with stage IV breast cancer. The systematic search of 4 databases (PubMed, Scopus, Embase and Cochrane CENTRAL) and manual search of references yielded 338 studies for screening. Two independent reviewers performed the initial title and abstract screening, and nine studies were chosen for retrieval and full text assessment. Their percentage of agreement was 70.2% and Cohen's kappa was 0.11. Finally, three reports were selected, abiding to the inclusion and exclusion criteria. Taken together, they included 124 patients. Each one of them utilized a different type of systemic treatment: chemotherapy, interleukin-2, endocrine therapy with tamoxifen. All reports were assessed for

risk of bias, by two independent reviewers, and there were some points in the study methodology that raised some concerns, particularly patient allocation, blinding of both participants and caregivers, adherence to therapy and blinding of the radiologists assessing tumor response. Furthermore, all three trials were conducted by the same research group in Italy, during the 1990's.

All three reports contained data on tumor response, for a total of 124 patients. The I^2 statistic was 0% indicating no observed heterogeneity among the studies. OR for tumor response was 2.91 (95% CI, 1.29-6.57, p = 0.01), significantly in favor of the melatonin group. For the melatonin group, ARR was 20% and the NNT was 5.0. Survival data were provided in only two reports with encompassing 117 patients, and the I^2 statistic was, again, 0%. OR for 1-year survival was 3.40 (95% CI, 1.63-7.08, p = 0.001), significantly in favor of the melatonin group. For the melatonin group, ARR was 29% and the NNT was 3.4.

7.4.1 PAPER IV: CERTAINTY OF EVIDENCE

In this systematic review and meta-analysis, we tried to investigate the role of melatonin, as an adjunct to systemic oncologic therapy in patients with stage IV breast cancer. We found only three studies including patients with metastasized breast cancer, although similar studies have been conducted including patients with other types of cancers. Our analysis on tumor response was conducted on 124 patients, and the 1-year survival analysis used data from 117 patients. As can be seen, the numbers are quite small to draw robust conclusions. Even though the effect sizes found are significant and in favor of the melatonin group, our results should be cautiously interpreted, given the fact that they are based on a small number of patients. In addition to the above, all studies were conducted by the same research group in Italy, almost 30 years ago. The lack of external validation by independent research group, may be viewed as a limitation. Furthermore, at that time, the methodology of randomized controlled trials was not as precisely delineated, as it is today. Nor was reporting as standardized. Consequently, there is some room for bias. It is for these reasons that the certainty of evidence of the meta-analysis is regarded as low, despite the statistically significant results and meticulous methodology in the implementation of the SR/MA.

8 DISCUSSION

8.1 PAPER I

The aim of the study was to investigate the effect of melatonin intake on survival of patients with early breast cancer within a large population cohort derived from the Swedish national registries. Even though melatonin exposure was associated with improved outcome for BCSS in the univariable analysis, the association did not persist after adjustment for known prognostic factors in the multivariable analysis.

The study represents, to the best of our knowledge, the largest study trying to investigate such an effect. It relies on data retrieved from a reliable source, the Swedish registry, in a country where melatonin, is mostly a prescription drug. The findings could reflect differences in the patients' health-seeking behaviors, comorbidity profiles or other unmeasured confounding factors. The small number of patients that had actually used melatonin, a mere 2.5%, represents an additional limiting factor. The real percentage however might be different since there are ways of obtaining melatonin without a prescription, e.g. by ordering through the internet or while travelling abroad in countries where it is available over the counter.

Furthermore, the limited duration of melatonin exposure, with most patients having taken it for a median of only 30 days, is unlikely to exert a biologically meaningful effect on long-term survival outcomes. It could be advocated that a more prolonged exposure could, theoretically at least, provide a different or statistically more robust outcome either way. With that meaning that even though the effect, favorable or unfavorable, concerning survival, is unknown, the longer duration of the exposure could warrant more confidence in the results. This of course could raise questions concerning the dose as well as the duration of the treatment, but also its relation to the timing of diagnosis, surgery and adjuvant therapy. The latter is of particular importance since intake of melatonin before the diagnosis could assess its effect on the risk of developing breast cancer, while intake after the diagnosis and until the time of surgery would mean that melatonin had a "window of opportunity" to exert its effects on the tumor and the circulating tumor cells. Finally, its concomitant administration during adjuvant treatment, would contaminate the results with any synergy between melatonin and chemotherapy, radiotherapy or hormonal therapy, if such a compounding effect really exists.

Despite the lack of a significant association in the current study, the plausibility of melatonin having a role in the treatment of early breast cancer still remains given the amount of preclinical and experimental evidence from research conducted so far. The absence of well-designed randomized controlled trials investigating the effect of melatonin in early breast cancer, represents a gap in the current literature and emphasizes the need for more rigorous investigation. A clinical trial on the effect of melatonin on the survival of patients with uveal melanoma is currently in progress (215). A similar pilot study could be designed for breast cancer, with specific pre-defined outcomes and after careful considerations of dosage, treatment duration and timing in relation to the standardized treatment.

8.2 PAPER II

The study investigated the clinical relevance of the MT1 melatonin receptor expression in breast cancer tissue. No significant associations were found between its expression and known clinicopathological parameters or survival outcomes. These findings are in accordance with some, but not all previous similar studies, indicating the inconsistency in the current evidence base. Such discrepancies may reflect methodological differences in immunohistochemical assessment, inexperience of the laboratory in working with that particular antibody, as well as lot-to-lot variability of the antibody. Individual assessment biases as well as differences in the scoring systems could also contribute.

The study material included only invasive ductal breast carcinoma samples, from post-menopausal women, in an effort to revisit the Cohen hypothesis concerning hormone receptor-positive breast cancer. With the majority of breast cancers in women over 60 years of age, being hormone receptor-sensitive, we hypothesized that the expression of MT1 could be in some way correlated with tumor characteristics and/or survival. The results did not confirm the hypothesis, mainly due to the lack of a correlation between ER and MT1 expression. It could therefore be argued that the expression of the receptors in breast carcinoma is an amplification of their normal expression for yet unknown reasons. Since normal breast tissue expresses both MT1 and MT2, the question remains concerning the preference for the sole expression of MT1 in breast malignant tumors.

In conclusion, there is currently insufficient evidence to argue for the use of melatonin receptors as prognostic biomarkers, with the probable exception of triple negative breast cancer, where a study has shown that MT1 expression

was correlated with lower Ki67 and improved patient survival (216). Perhaps a more inclusive study with various histological types, both ER positive and negative, and from patients from all age groups, in sufficient numbers to permit subgroup analysis, could shed more light to the question.

8.3 PAPER III

This nationwide cohort study, which is the largest to investigate the effect of visual impairment on cancer risk, included Swedish men and women aged 40 years or older, with visual impairment, and their matched controls. Overall, individuals with visual impairment had a higher CCI score and a greater prevalence of cancer diagnoses in the five years preceding the study, indicating that visual impairment is more an indicator of health vulnerability and less a protective marker against malignancy.

During the follow-up, visual impairment was associated with significantly elevated overall cancer risk. Furthermore, significant risks were observed for many of the site-specific malignancies examined, whereas the risk was comparable for others. There were no significant differences between the sexes. Increasing age conferred augmented risk in both the visual impairment and the seeing parts of the cohort, in accordance with epidemiological evidence that age, but also comorbidities are principal determinants of cancer risk. The increased risk of brain cancer, confirmed by another similar study, may reflect reverse causality or genetic conditions affecting both visual impairment and cancer.

The observed associations could be explained by various confounding factors. Those related to the visual system include age-related macular degeneration, and diabetic retinopathy. Factors related to the individuals include age, obesity and comorbidities. Finally, lifestyle factors concerning diet, physical exercise, parity, smoking and alcohol consumption may also contribute to both visual impairment and neoplasia. In a similar way, the elevated risk may partly reflect increased healthcare utilization among individuals with visual impairment, with more frequent diagnostic assessments; an effect commonly referred to as surveillance bias. Conversely, existing medical conditions such as visual impairment may divert attention from early signs and symptoms of malignancy.

The findings also call attention to the reciprocal relationship between visual impairment and socioeconomic status. Lower socioeconomic status is

associated with a higher prevalence of visual impairment, mainly because of reduced access to ophthalmologic care, eye screening and refractive correction, cataract surgery and adequate management of conditions such as glaucoma and diabetic retinopathy. Furthermore, individuals of lower socioeconomic status are more likely to be exposed to occupational hazards, or infectious diseases that impair vision. On the other hand, visual impairment can contribute to lower socioeconomic status by limiting educational advancement, employment opportunities and career advancement. These in turn might impede access to health information, cancer screening and preventive services.

Overall, the increased incidence of cancer in the visual impairment is multi-factorial, representing a combination of genetic, biological and social parameters, with the relative contribution of each being hard to quantify. However, the results highlight the existence of inequities among individuals with visual impairment, in comparison to the rest of the population, calling attention to the need for reforms in public health strategies and health care services, to ensure equity and inclusion.

In the present study, visual impairment was used as a potential proxy for unimpeded melatonin production. Earlier studies proposed a protective role of visual impairment in cancer incidence. However, accumulating evidence, especially pertaining to light perception in individuals with blindness, and the present findings do not support melatonin as a plausible explanatory mechanism.

8.4 PAPER IV

The SR/MA undertaken indicates that the addition of melatonin to standard oncological treatment in patients with stage IV breast cancer is associated with a significantly improved tumor response and improved 1-year survival, with large effect sizes for both outcomes. Furthermore, the examined studies indicate a reduction in treatment-related toxicity, suggesting a compound benefit in terms of both efficacy and tolerability for that particular group of patients.

However, the findings should be interpreted with caution, since the number of included studies is small, with limited sample sizes and some methodological considerations could be raised concerning the allocation procedures used, the absence of placebo controls and the lack of blinding of both participants and assessors. Furthermore, all studies are conducted by the same research group,

which has performed extensive research with melatonin during the 1990's, indicating a possible conviction of the researchers in the utility and efficacy of melatonin in the field of oncology. This could potentially introduce confirmation bias in the interpretation of the results, not so much in the outcomes measured, which are fairly objective (tumor response, 1-year survival), as in the reporting of the treatment side-effects. An additional factor hindering the generalizability of the findings is the use in one study, of interleukin-2, which is not currently used in the treatment of that patient group. What's more, the relatively high doses of melatonin used in the trials, raise questions regarding optimal dosing and safety, despite the generally favorable safety profile reported in systematic reviews.

In conclusion, while the available results indicate a potential benefit of melatonin in improving outcomes and mitigating adverse effects in patients undergoing palliative treatment for stage IV breast cancer, it remains insufficient to advocate their routine clinical implementation. It does however justify the need for contemporary, well-designed randomized controlled trials in order to confidently advocate the incorporation of melatonin to the treatment of this patient population, if the results are favorable.

9 CONCLUSION

The present thesis aimed to explore the role of melatonin in breast cancer within different conceptual frameworks. The extent to which this was achieved is analyzed in the Results and Discussion. Nevertheless, as this investigation is coming full circle, there are a few concluding remarks.

The effect of melatonin intake was found to be significant in the univariable analysis of BCSS but disappeared in multivariable analysis. No effect was found for OS. This indicates that melatonin ingestion has no impact on the survival of breast cancer patients. However, the exposure was limited, and the analysis did not provide information on the ingestion of melatonin in relation to time of diagnosis and treatment. A separate analysis with that information, could help in the clarification of the issue.

Expression of the MT1 melatonin receptor in ER positive/HER2 negative invasive ductal breast carcinoma and its axillary metastases was not found to be associated with known prognostic and predictive factors, OS or BCSS. This indicates that the overexpression of MT1 in breast cancerous tissue should be investigated from a different viewpoint. Its association with response to antihormonal therapy could be such an alternative.

Visual impairment was found to be associated with an increased incidence of cancer of various types, including breast. This study constitutes the larger of its kind, with 1:5 matching for age, sex and year of inclusion and adjustment for previous cancers. This, in conjunction with the robustness of the statistical analysis affords confidence for the results, but the presence of multiple confounding factors does not allow the delineation of direct causal relationships.

The SR/MA has yielded significant results on enhanced tumor response and prolonged 1-year survival with the addition of melatonin to systemic oncologic therapy in patients with stage IV breast cancers. This could serve as a starting point to further investigate its effect in patients receiving current therapeutic regiments and even immunotherapy. An additional approach would be to investigate the effect of melatonin addition to oncologic therapy across the different intrinsic types of breast cancer in the same patient group.

10 FUTURE PERSPECTIVES

10.1 POTENTIAL USE OF MELATONIN IN ADJUVANT BREAST CANCER TREATMENT

Melatonin could have a place in the adjuvant therapy of early breast cancer, taking advantage of its oncostatic, immunomodulatory and anti-estrogenic effects. Between 2017 and 2024, new research has emerged examining the effect of melatonin supplementation to known chemotherapeutic and immunotherapeutic agents in the preclinical setting (217). Combination with paclitaxel, potentiated its cytotoxic effect in both ER positive and triple negative breast cancer cells (218). It synergistically increased the anti-proliferative and apoptotic effect of docetaxel on MCF-7 cells (219). Two studies showed increase in apoptosis and a synergistic cytotoxic effect of doxorubicin and melatonin in both ER positive and triple negative breast cancer cell lines (220, 221). Finally, three different experiments examined the combination of lapatinib, neratinib and apatinib with melatonin, reporting increased cytotoxicity in HER2 positive breast cancer cell lines (222-224).

An animal study showed that melatonin helped overcome LAN-induced resistance to doxorubicin (225) raising the question of temporal alignment of therapy, in relation to the patient's circadian rhythm, along with the problem of acquired resistance to therapy.

Notably, melatonin was shown to increase the sensitivity of human breast cancer cells to radiation (226, 227). In addition to that, its role has been investigated in the management of radiation-induced skin toxicity. In a prospective randomized placebo-controlled double-blind study, of women operated with breast-conserving surgery and undergoing radiation treatment, participants using the melatonin-containing mixture on the irradiated area, had significantly reduced radiation dermatitis than the controls who used placebo (228). In a similar study evaluating the quality of life, patients using the melatonin-containing cream, reported significantly lower breast symptom scores (229).

Melatonin's dual capacity as both SEEM and SERM could be leveraged therapeutically in cases of hormone receptor positive breast cancer. Current

ongoing research is focusing on melatonin-tamoxifen conjugates, aiming at potentiating the effects of endocrine therapy, while reducing adverse effects and overcoming resistance. Some of the conjugates have shown promising results in preclinical assessments (230-232).

Taken together, research evidence indicates that melatonin could be used as an adjunct to already existing therapeutic protocols, to increase their potency and possibly help overcome resistance.

10.2 POTENTIAL USE OF MELATONIN IN PALLIATIVE BREAST CANCER CARE

Melatonin has been investigated as an adjuvant agent in the palliative care of patients with breast cancer. Its reasonably safe profile and low cost, in combination with its oncostatic abilities, make it a promising contributor to the care of patients with stage IV breast cancer. Evidence suggests that melatonin may alleviate cancer-related fatigue and the adverse effects of chemotherapy (233). Randomized trials indicate that the addition of melatonin to oncologic treatment, in patients with advanced breast cancer, significantly improved (234-236) tumor response and 1-year survival, while alleviating chemotherapy-induced side-effects .

Furthermore, the demonstrated ability of valproic acid to enhance MT1 receptor expression in both hormone receptor-positive and negative breast cancer cells lines, indicates an area probably worth exploring (237, 238).

10.3 POTENTIAL USE OF MELATONIN IN BREAST CANCER PREVENTION

Chronodisruption has been shown to increase the risk of breast cancer through exposure to LAN or shift work. The effect of LAN could be counteracted with behavior modification, better consideration of working schedules, and the installation of blue light filtering devices. Investigating the use of blue filtering lenses on sleep quality, a recent Cochrane review yielded mixed results, while commenting on the heterogeneity of the study populations (239). Nevertheless, the use of screens before bedtime is associated with later bedtimes and sleep disruption in a recent cross-sectional study (240). Some have even argued that women carrying BRCA mutations should minimize screen exposure before bedtime (241).

Melatonin's antiestrogenic properties could support preventive strategies in individuals with risk factors such as obesity (increased aromatase activity), or night shift work (242).

In conclusion, melatonin demonstrates antiproliferative, pro-apoptotic anti-angiogenic and immunomodulatory properties that could support its role as a potential chemopreventive agent. Despite the mechanistic and experimental evidence that exists, clinical data remain incomplete and even though pre-clinical research suggests a possible role for melatonin in the prevention of breast cancer development, large-scale prospective randomized trials are required to attest its efficacy and optimal use.

11 EPILOGUE: FINAL REFLECTIONS

In the distant past, Aristotle said that “Nature does nothing in vain”. While searching the bibliography for melatonin, learning about the additional functions of the “hormone of darkness”, this would echo repeatedly, triggering the curiosity to learn more. And this culminated in the present thesis trying to investigate the role of melatonin in breast cancer in a scientific way.

We tried to approach the question from different angles. We looked for a possible protective effect between melatonin ingestion and survival in patients with early breast cancer, which was not confirmed in the retrospective registry study presented in Paper I. The investigation of MT1 receptor expression in breast cancer gave no “favorable” results either, finding no association between MT1 expression and clinicopathological parameters or survival, as shown in Paper II. Visual impairment was associated with increased cancer incidence, with multiple confounding factors contributing to the results and melatonin not being implicated, as explained in Paper III. The final undertaking, a systematic review, showed that the addition of melatonin to oncological treatment conferred improved tumor response and survival in patients with stage IV breast cancer. At long last a “positive” result, based however on a small number of patients, participating in an even smaller number of studies. Results that one could easily argue are not enough to urge the inclusion of melatonin in clinical practice.

So, the question arises if the theory was wrong from its inception. It could be that melatonin does not have, after all, a protective role against breast cancer. Cohen’s hypothesis could be what it is, a mere hypothesis, a speculation based on coincidental facts with no underlying true relationships. According to K. Popper, a scientific theory cannot be verified, only falsified. The scientific inquiry should not aim towards verifying hypotheses, but rather strive to disprove them, exposing their weaknesses and vulnerabilities. Science advances by identifying and eliminating errors. And the present thesis has managed to identify errors, which might stem from the way the issue in question was approached. In Paper I the intake of melatonin was not examined in relation to the timing of diagnosis and for most of the patients, treatment duration was short. Melatonin might have synergized with adjuvant therapy, if taken after surgery. Assessment of receptor expression was confined to Luminal breast cancer in postmenopausal women. This also confers a limitation. Finally, the SR/MA identified only three studies with stage IV

breast cancer, although there are similar studies including patients with other types of cancers with favorable results on the meta-analyses.

It is important that the results are communicated to the scientific community so that other scientists will not continue on the same path, but rather proceed, if interested, along a different route. Research on melatonin, its effects and potential use in the field of breast cancer remains active, driven by hypotheses and even inferences derived from preclinical evidence. Together with its favorable safety profile and low cost, it represents an attractive option that could be used as an adjunct in the treatment of breast cancer. Before abandoning the theory altogether, it may be reconsidered from a different perspective. The Swedish Registries constitute a comprehensive and reliable repository of retrievable information from where we can investigate the intake of melatonin and the risk of developing breast cancer. Neo-adjuvant treatment and time from diagnosis until surgery provide windows of opportunity for assessing the effects of melatonin in vivo, by focusing on the assessment of tumor response and on differences in gene expression of important markers (receptor expression, proliferation), before and after treatment.

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USE OF GENERATIVE AI

No generative AI tools were used in the preparation of this thesis.

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