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Genetic and psychopathological characteristics of patterns of nocturnal symptoms of insomnia

An epidemiological perspective using data from the HUNT3 study

NTNU
Norwegian University of Science and Technology
Thesis for the Degree of
Philosophiae Doctor
Faculty of Medicine and Health Sciences
Department of Mental Health

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"*Science, my boy, is made up of mistakes; but of mistakes which lead to the discovery of truth.*"

Jules Verne

Journey to the Centre of the Earth

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Acknowledgments

The doctoral project summarized in this thesis was conducted at the Department of research and development (AFFU) of the Institute of Mental Health, at the Norwegian University of Science and Technology.

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Finally, I would like to thank my dearest Tino for being there, steadily holding my hand, always.

List of papers

Paper 1

Bragantini D, Sivertsen B, Gehrman P, Lydersen S, Güzey IC. *Variations in circadian genes and individual nocturnal symptoms of insomnia. The HUNT study*. Chronobiology international. 2019; 36(5):681-8.

Paper 2

Bragantini D, Sivertsen B, Gehrman P, Lydersen S, Güzey IC. *Genetic polymorphisms associated with sleep-related phenotypes; relationships with individual nocturnal symptoms of insomnia in the HUNT study*. *BMC Medical Genetics*. 2019; 20:179.

Paper 3

Bragantini D, Sivertsen B, Gehrman P, Lydersen S, Güzey IC. *Differences in anxiety levels among symptoms of insomnia. The HUNT study*. Sleep Health. 2019; 5(4):370-375.

Paper 4

Bragantini D, Sivertsen B, Gehrman P, Lydersen S, Güzey IC. *Epidemiological differences in levels of depressive signs among nocturnal symptoms of insomnia; results from the HUNT study*. Sleep science and practice. 2020; 4(7).

Summary

Background and objectives

Chronic insomnia is a sleep disorder characterized by diurnal and nocturnal symptoms. The night-time disturbances specified by the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) are: difficulties in falling asleep (sleep onset insomnia), frequent awakenings during the night (maintenance insomnia) and early morning awakenings (terminal insomnia). One aspect often ignored in insomnia research is that these symptoms may appear in different combinations. Hypothetically, although related, these symptoms may have different characteristics and causes. Two widely investigated aspects of insomnia are its relationship with other psychological disorders, like anxiety and depression and their genetic background. Unfortunately, neither of these aspects has been studied considering nocturnal symptoms of insomnia separately. The aim of this PhD project was to investigate whether the nocturnal symptoms of insomnia should be considered equivalent manifestations of the same disorder or separate entities with individual genetic backgrounds and relationships to psychopathological symptoms.

Material and methods

The individuals selected to be included in all four studies described in this thesis were selected among individuals who participated in the third round of the Nord-Trøndelag Health Study (the HUNT3 study, Norway). Information about insomnia, psychological health and genetic variations for the participants were obtained from the HUNT data and biobank. In each study, the presence of each nocturnal symptom of insomnia was evaluated using a Likert-like scale ("Never", "Sometimes", "Several times a week"). Participants who answered "Several times a week" to at least one question were selected as cases, while those who answered "Never" to all question were defined as controls. For the first two studies, we selected 6029 participants with genetic data, 3577 cases and 2452 controls. In study 3 and 4 we had data for 7933 individuals, 4317 cases and 3616 controls.

In study 1, multinomial regression was used to assess the association among all seven possible combinations of symptoms of insomnia and 73 single nucleotide polymorphisms (SNPs) selected on nine core circadian genes (*PER1, 2, 3, CRY1, 2, TIMELESS, CLOCK, REV-ERBa, ARNTL*).

In study 2, multinomial regression was used to test the associations among all possible patterns of insomnia symptoms and 59 SNPs previously reported as associated with sleep traits.

In study 3 we reported means scores for the anxiety subscale of the Hospital Anxiety and Depression Scale (HADS) among the patters of symptoms of insomnia and compared them using ANOVA.

Finally, using the same methodology of study 3 in study 4 we focused on differences on the depression subscale of HADS among the different patterns of symptoms.

Results

In study 1, 25 of the SNPs in circadian genes were significantly associated with patterns of symptoms of insomnia. We observed that the majority of SNPs on gene *PER3* were associated with reporting all three symptoms, those on gene *CRY* with early morning awakenings and those on *ARNTL* with sleep onset insomnia. Nevertheless, no association remained significant after applying correction for multiple testing.

In study 2, 16 SNPs selected from previous studies on sleep traits were associated with several combinations of symptoms of insomnia. However, none of them remained significant after applying correction for multiple statistical testing.

In study 3, mean HADS-A score was statistically different among several insomnia symptoms ($p < .001$). Participants reporting sleep maintenance insomnia only (M = 4.5, SD = 3) had the lowest mean anxiety score while those experiencing all three symptoms had the highest (M = 6.8, SD = 4.3). Persons reporting only sleep onset insomnia had the highest mean HADS-A score among respondents with just one

symptom (M = 5.8, SD = 3.7). Overall, difficulties falling asleep seemed to play a decisive role in rising HADS-A levels.

In study 4, we found only moderate differences in HADS-D levels among patters of symptoms of insomnia. Persons who reported all three symptoms of insomnia had the highest HADS-D mean score (M=5.2, SD=3.6), however the mean did not differ significantly from those who experienced sleep onset insomnia combined with terminal insomnia (M=5.4, SD=3.4). Correspondingly, participants who reported only sleep maintenance insomnia had the lowest mean score (M=3.4, SD=2.9).

Conclusions

Anxiety levels as measured by HADS-A were the only significant and relevant element that differed among patterns of symptoms of insomnia. In particular, sleep onset insomnia was the symptom that displayed the highest levels of anxiety, both alone and when occurring with other symptoms. Even if we did not find any statistically significant association among the phenotypes and the genetic markers we selected, we cannot exclude that symptoms of insomnia may have different genetic backgrounds. Despite the lack of statistical significance, the results may be indicative of the presence of influence of the investigated genes on different symptoms. The assumption arises from the almost exclusive association of some genes (*PER3, CRY1, CRY2* and *ARNTL)* with specific symptoms and the plausible biological role of some genes already associated with sleep phenotypes. Differences in depression levels were not clinically significant among the symptoms but measuring tool with higher precision than HADS-D may reveal these differences in future studies.

Norsk sammendrag

Bakgrunn og mål

Kronisk insomni (søvnløshet) er en søvnforstyrrelse preget av daglige og nattlige symptomer. I henhold til *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5)* finnes det tre typer nattlige symptomer: vanskeligheter med innsovning, hyppige oppvåkninger i løpet av natten og tidlig oppvåkning om morgenen. Et aspekt som ofte blir oversett i forskning på insomni er at disse tre symptomene kan forekomme i ulike kombinasjoner. Selv om de er relatert, kan disse symptomene hypotetisk sett både ha forskjellige egenskaper og årsaker. To mye undersøkte aspekter ved insomni er forholdet til andre psykologiske lidelser, som angst og depresjon og deres genetiske bakgrunn. Dessverre har ikke disse aspektene blitt studert på en måte som tar hensyn til de ulike nattlige symptomene hver for seg. Målet med dette doktorgradsprosjektet var å undersøke om nattlige symptomer på insomni bør betraktes som likeverdige manifestasjoner av den samme lidelsen eller separate enheter med en individuell genetisk bakgrunn og forhold til psykopatologiske symptomer.

Materialer og metoder

Individene som ble valgt for å bli inkludert i alle de fire studiene som er beskrevet i denne oppgaven, ble valgt blant individer som deltok i tredje runde av Helseundersøkelsen i Nord-Trøndelag (HUNT3-studien, Norge). Informasjon om insomni, psykologisk helse og genetiske variasjoner for deltakerne ble hentet fra HUNT-data og biobanken. I hver studie ble tilstedeværelsen av hvert nattlig symptom på insomni evaluert ved å bruke en Likert-lignende skala ("Aldri", "Noen ganger", "Flere ganger i uken"). Deltakere som svarte “Flere ganger i uken” på minst ett spørsmål ble valgt som tilfeller, mens de som svarte “Aldri” på alle spørsmål ble definert som kontroller. For de to første studiene valgte vi 6029 deltakere med genetiske data, 3577 tilfeller og 2452 kontroller. I studie 3 og 4 hadde vi data for 7933 individer, 4317 tilfeller og 3616 kontroller.

I studie 1 ble multinomial regresjon brukt for å vurdere sammenhengen blant alle syv mulige kombinasjoner av symptomer på insomni og 73

enkeltnukleotidpolymorfismer (single nucleotide polymorphisms - SNPs) valgt på ni cirkadiske gener (*PER1, 2, 3, CRY1, 2, TIMELESS, CLOCK, REV-ERBa, ARNTL)*.

I studie 2 ble multinomial regresjon brukt for å teste sammenhengene mellom alle mulige mønstre av insomnissymptomer og 59 SNPs tidligere rapportert som assosiert med søvnegenskaper.

I studie 3 rapporterte og sammenlignet vi gjennomsnittsresultater for angstens delskala i Hospital Anxiety and Depression Scale (HADS) blant utbredelsen av symptomer på insomni ved bruk av ANOVA.

Til slutt, ved å bruke den samme metodikken som i studie 3, fokuserte vi i studie 4 på forskjeller på depresjonens delskala i HADS blant de forskjellige symptommønstrene.

Resultater

I studie 1 var 25 av SNPs i cirkadiske gener signifikant assosiert med mønstre av symptomer på insomni. Vi observerte at flertallet av de signifikante SNPs på gen *PER3* var assosiert med rapportering av alle tre symptomene, de på genene *CRY* med oppvåkninger om morgenen og de på *ARNTL* med vanskeligheter med innsovning. Likevel var det ingen av disse sammenhengene som forble signifikante etter korreksjon for multiple sammenlikninger.

I studie 2 var 16 SNPs, valgt fra tidligere studier på søvnegenskaper, assosiert med flere kombinasjoner av symptomer på insomni. Ingen av dem forble imidlertid signifikante etter å ha brukt korreksjon for flere statistiske tester.

I studie 3 var de gjennomsnittlig HADS-A-resultatene statistisk forskjellige blant flere symptomer av insomni (p <0,001). Deltakere som bare rapporterte hyppige oppvåkninger i løpet av natten hadde det laveste gjennomsnittlige resultatet på angst (M = 4,5, SD = 3), mens de som opplevde alle tre symptomene hadde det høyeste (M = 6,8, SD = 4,3). Personer som bare rapporterte vanskeligheter med innsovning hadde det høyeste gjennomsnittlige HADS-A-resultatet blant respondentene med bare ett symptom (M = 5,8, SD = 3,7). Totalt

sett virket det som at vanskeligheter med å sovne spiller en avgjørende rolle for å øke HADS-A-resultatene.

I studie 4 fant vi bare moderate forskjeller i HADS-D-resultater blant utbredte symptomer på insomni. Personer som rapporterte alle tre symptomene på insomni hadde det høyeste gjennomsnittet i HADS-D-resultater (M = 5,2, SD = 3,6), men gjennomsnittet skilte seg ikke signifikant fra de som opplevde vanskeligheter med innsovning kombinert med tidlig oppvåkning (M = 5,4, SD = 3,4). Tilsvarende hadde deltakere som kun rapporterte hyppige oppvåkninger i løpet av natten det laveste gjennomsnittlige resultatet (M = 3,4, SD = 2,9).

Konklusjoner

Angstnivåer målt ved HADS-A var det eneste signifikante og relevante elementet som skilte seg blant mønstre av symptomer på insomni. Spesielt var vanskeligheter med innsovning som var det symptomet som viste de høyeste nivåene av angst, både alene og når de oppsto i kombinasjon med andre symptomer. Selv om vi ikke fant noen statistisk signifikant sammenheng mellom fenotypene og de genetiske markørene vi valgte, kan vi ikke utelukke at symptomer på søvnløshet kan ha ulik genetisk bakgrunn. Til tross for mangelen på statistisk betydning, kan resultatene være en indikasjon på tilstedeværelsen av påvirkning av de undersøkte genene på forskjellige symptomer. Antagelsen stammer fra den nesten eksklusive sammenhengen mellom noen gener (*PER3, CRY1, CRY2 og ARNTL*) og spesifikke symptomer og den sannsynlige biologiske rollen til noen gener som allerede er assosiert med noen søvnfenotyper. Forskjeller i depresjonsnivåer blant symptomene var ikke klinisk signifikante, men måleverktøy med høyere presisjon enn HADS-D kan avsløre disse forskjellene i fremtidige studier.

Abbreviations

ADAMTS14: ADAM Metallopeptidase with Thrombospondin Type 1 Motif 14

AK5: Adenylate Kinase 5

APH1A: Aph-1 Homolog A, Gamma-Secretase Subunit

ARNTL: Aryl hydrocarbon receptor nuclear translocator-like protein 1

ATP: Adenosine triphosphate

BMAL1: Brain and Muscle ARNT-Like 1

BPD: Bipolar Disorder

CACNA1C: Calcium Voltage-Gated Channel Subunit Alpha1 C

CACNA2D3: Calcium Voltage-Gated Channel Auxiliary Subunit Alpha 2 Delta 3

CEU: Utah Residents (CEPH) with Northern and Western European Ancestry

DSM-5: Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition

EBF3: Early B-Cell Factor 3

EEG: Electroencephalogram

ESRRG: Oestrogen Related Receptor Gamma

FDR: False Discovery Rate

fMRI: functional Magnetic Resonance Imaging

GWAS: Genome Wide Association Study

HADS: Hospital Anxiety and Depression Scale

HUNT: Helse Undersøkelse Nørd-Trøndelag (Nord-Trøndelag Health Study)

ICD-10: International Classification of Diseases-10

ICDS: International Classification of Sleep Disorder

MAF: Minor Allele Frequency

MDD: Major Depression Disorder

MEIS1: Meis Homeobox 1

MID: Minimal Important Difference

OPCML: Opioid Binding Protein/Cell Adhesion Molecule Like

PAX8: Paired Box 8

PER2: Period Circadian Regulator 2

PIN1: Peptidylprolyl Cis/Trans Isomerase, NIMA-Interacting 1

RANBP5: Uridine Phosphorylase 2

SA: Sleep Aprnoea

SATB2: Special AT-Rich Sequence-Binding Protein Homeobox 2

SNPs: Single Nucleotide Polymorphisms

SOL: Sleep Onset Latency

TOX3: TOX High Mobility Group Box Family Member 3

UPP2: Uridine Phosphorylase 2

WASO: Wake After Sleep Onset

1. Introduction

"*Insomnia is an affection which is trying to both physician and patient alike, and many are the remedies which have been recommended for its cure. The latest of these is the peanut, eaten ad libitum just before retiring. A member of the clergy reports success with the peanut after having tried other means without results.*"

(Science, 10 May 1889).

The remedy for insomnia presented in this communication sounds grotesquely old-fashioned but the description of insomnia remains valid even 130 years after its publication. Often overlooked, insomnia is indeed vexing for those who experience it: "Only the insomniac looks on with open eye, like a cadaver who forgot to die" as expressed by Hungarian writer Gyula Krúdy.

Research on insomnia advanced a great deal from the days of peanuts in large doses before going to bed. Indeed, nowadays research on insomnia is approached from numerous sides of biology, medicine and psychology. However, even if effective therapeutic interventions for insomnia are available, continuing in increasing our understanding of insomnia will allow designing more effective, targeted interventions.

Although in recent years research on insomnia has received much attention, one aspect that is been relatively much ignored is the presence of three nocturnal symptoms of insomnia corresponding to onset, maintenance and early termination of sleep. These are considered equivalent in clinical practice when setting a diagnosis[2] but may have different causes and lead to different implications and consequences.

From a methodological perspective, characterization of different symptoms is common in other field of medicine. Researchers have exploited tools from epidemiology and molecular genetics to investigate differences among symptoms of several medical conditions such as cancer[3,4], gastrointestinal syndromes[5,6], delirium[7], major depressive disorder (MDD)[8,9], schizophrenia[10,11] to cite only a few.

This approach has led to improvement in the taxonomy of these diseases[3,5-8,10,11] and identification of targeted interventions[4,9]. Approaching insomnia in the same way may allow distinct characteristics of each symptom to emerge and to be addressed specifically.

The idea to approach symptoms of insomnia separately is not however merely a methodological issue. Onset, maintenance and termination insomnia could be the pathological expression of the three biological diverse physiological main phases of sleep: onset, maintenance and termination. Opening the "diagnostic box" marked insomnia and looking at the inside through the lenses of sleep physiology may show that the symptoms are biologically diverse.

1.1 The importance of sleep

"Birds do it, bees do it, even educated fleas do it…" sings a popular song claiming that these animals all "fall in love". There is no scientific evidence of these organisms experiencing romance, but for sure they all do something else, they sleep.

Sleep (or sleep-like rest states) is a phenomenon that is conserved from sea sponges to insects, from fish to mammals[12]. As all these beings need food to survive, sleep is also among the vital needs. Lack of sleep is not only deleterious to cognitive functions[13] but it affects other physiological functions such as core temperature and brain development[14].

Lack of sleep creates both physiological and psychological stress, but at the same time in an evolutionary scenario, sleep might appear as a disadvantage. Losing consciousness leaves animals in an unfavourable position towards predators. Still as sleep is widely conserved in some forms through the animal kingdom this disadvantage seems to be a favourable compromise towards life[12].

So, why do we sleep? This question has produced several hypotheses throughout the last two centuries. All theories of sleep are the product of the state of the art of physiology and neuroscience of the time. However, sleep is a complicated

process, as many other cerebral functions and the question has yet to find a unanimous answer.

After early attempts to determine the cause of sleep, the past 120 years have seen the birth of several molecular and neurocognitive theories. The most recent theories focus especially on cellular metabolism and looks at accumulations of catabolites and scarceness of fundamental molecules as possible starting signal for sleep. During waking time, the brain consumes glycogen and molecules at a higher rate than it can synthetize. Immobility, synchronized neuronal firing and reduced processing of sensory stimuli typical of sleep, are the way of the brain of running in "power-saving mode". Stand-by of so many functions would allow cells to replenish the stock of macromolecules[15], restore glycogen levels[16] and to operate structures and DNA maintenance[17].

Information processing and synaptic plasticity to form long term memories are another set of theories that are well supported[18], but seems to explain secondary processes rather than the homeostatic function[I] of sleep.[17] At a macroscopic level sleep deprivation manifest itself as decreased cognitive and psychological functions such as attention, reactivity, emotional processing, memory among others.[20] The wide range of functions that are affected by lack of sleep, could suggest that the purpose of sleep is dictated by basic cellular needs (ex: metabolic maintenance) more than specific brain functions (ex: memory formation) [17].

1.1.1 Processes regulating sleep

The theories of sleep centred on molecular and cellular turnover could have come closer answering the question "why do we sleep?" in the sense of "what provoke *the need to sleep*?"

Sleep-need, also called *sleep pressure* or *Process S*, is one of the two major regulators of sleep. As mentioned earlier, sleep is a homeostatic mechanism. Sleep-

[I] **Homeostasis**: "*The maintenance of a dynamically stable state within a system by means of internal regulatory processes that tend to counteract any disturbance of the stability by external forces or influences […]*" 19. In. *Oxford English Dictionary*: Oxford University Press; 2019.

need increases throughout the day, it "worsens" at night and improves after a night of sound sleep[1,21]. The increased concentration of Adenosine (Ado) in the extracellular space around neurons is one likely factor that constitute sleep pressure. Adenosine is produced by the degradation of molecules of Adenosine triphosphate (ATP)[II] and therefore is a by-product of cellular metabolism[22].

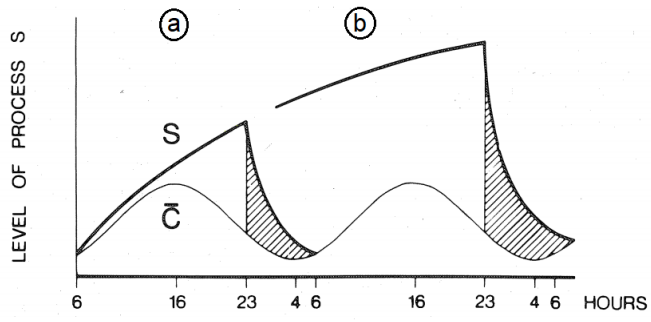

Figure 1. Process S (bold line) and Process C, or circadian rhythmicity (thin line). Sleep time in banded areas. A: a normal sleep-wake cycle, where sleep pressure increases throughout the day and decreases with sleep. B: in the absence of sleep, sleep pressure would increase. Adapted from Borbély. Hum Neurobiol. 1982;1(3):195-204.

The second process regulating sleep involves timing (Process C). Several biological phenomena run on a periodic schedule, and organisms follow this plan without the need of calendars or clocks. They respond to environmental cues such as sunlight intensity, temperature and nutrients to regulate the timing of many physiological functions. Fertility and migration are examples of events that re-occur with several days or months in between. Some biological functions follow a periodic "agenda" of about twenty-four hours and for this reason are defined "*circadian*" (from Latin "*circa"*, "about" and "*dies"*, "day").

The major entraining factor for circadian rhythm is light, in particular in the blue wavelength. When light is perceived by the retina, a signal is sent to the optic nerve to the neurons of the Superchiasmatic Nucleus (SCN) that constitutes the so-called *master clock*[23].

[II] **Adenosine triphosphate (ATP):** "a nucleotide consisting of adenosine and a triple phosphate unit, the hydrolysis of which is a source of energy for numerous physiological processes, and which also participates in many synthetic reactions requiring a phosphoric, adenosyl, or adenyl residue". 19. Ibid.

The signal is delivered to the nuclei of these neurons where a class of genes, also denominated, circadian, initiate a negative feedback loop. Light induces transcription of gene RORs. This induces the transcription of genes *ARNTL* and *CLOCK*, the products of which couples and work as transcription factors for *PER*, *CRY* and *REV* genes. Throughout the day, the concentration of the products of these genes increase and work as transcription factors for many genes and as repressors for their own transcription. *PER* and *CRY* couples impede the action of the *ARNTL-CLOCK dimer*. *REV* activates the same procedure towards RORs. This molecular loop is only a limited fraction of the complex system created by numerous circadian genes[24].

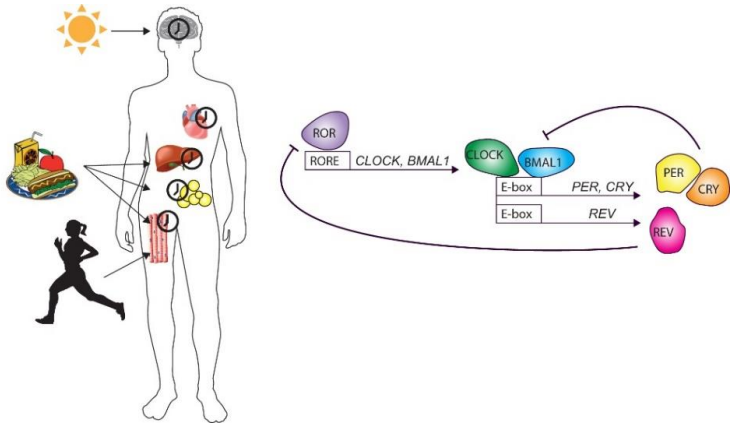

Figure 2: Master clock and peripheral oscillators (left) and the core components of the "molecular clock" (right). Figure from De Goede et al. J Mol Endocrinol. 2018 Apr;60(3):R115-R130

Process S and C interacts in a compensatory manner. When sleep pressure is high, circadian rhythmicity is overpowered and sleep can occur outside the usual times. However, when sleep pressure decreases under a certain threshold, the circadian clock regains control over the schedule[21]. Going to bed in the early morning usually does not allow sleeping throughout the day. High sleep pressure allows sleeping for only a few hours, before the SNC opposes the altered schedule.

1.1.2 Sleep physiology and architecture

The homeostatic and circadian mechanisms regulate a complex series of cellular and molecular events that allow onset, maintenance and termination of sleep. Moreover, sleep is constituted by different stages that reflect specific molecular, cellular and cerebral events.

The master clock located in the SCN is the timegiver for peripheral clocks located in several areas of the brain and other organs. Periodic firing from the master clock sets the pace for the other clocks that orchestrate the timing of cellular activity in their jurisdiction[25].

Sleep is timed through this system by temporally regulated release of several neurotransmitters and hormones[26]. It is hypothesized that release of GABA from SCN neurons is the starting point for sleep[27]. In an simplified model of sleep called the *flip-flop* or *switch model*[28], GABAergic neurons counteract the alertness inducing activity of orexin on principally the raphe nuclei (RN), the locus coeruleus (LC) and the tuberomammillary nucleus (TMN). These areas include serotonergic, noradrenergic and histaminergic neurons respectively. The neurotransmitters released by these neurons, serotonin, noradrenalin and histamine have wake inducing effects[26].

Progress in the neurophysiology of sleep has showed that the system is more complex than the name "flip-flop model" would let believe. Sleep is a "local" phenomenon of clusters of neurons in different areas of the brain. Increasing number of "sleeping" neuronal centres lead eventually to overall sleep[28,29] while selective reactivation of some areas explains several other aspects of sleep. Both molecular and electrophysiological evidence support this theory[28].

Tools such as electroencephalogram (EEG) and empirical observations allowed dividing sleep in different phases (REM, as for Rapid Eye Movements and non-REM, N-REM) [30]. These are characterized by fluctuations in release of neurotransmitters as confirmed by experimental and neuroimaging studies[30]. Characteristic EEG bands are not homogenous for all phases of sleep, indicating that different areas of the brain are independently active[31].

Sleep stages and electrophysiological parameters are subject to individual variability as many other traits. Genetic studies have identified some common polymorphisms that correlate to a natural predisposition to longer bouts of SWS.

In one of these studies, the long allele of a variable number tandem repeat[III] (VNTR) in the circadian gene *PER3* was associated with reduced cognitive performance after sleep deprivation[33]. The same effect was reported for carriers of a polymorphism in the gene *ADA*, which codes for Adenosine Deaminase, an enzyme that inactivate the sleep-inducing molecule, adenosine (Ado)[34].

1.1.3 Objective and subjective tools to investigate sleep

The EEG mentioned in the previous section is one objective tool used to collect information on cerebral activity. Several other tools are also used in research and clinical settings to investigate sleep and its affections.

1.1.3.1 Polysomnography

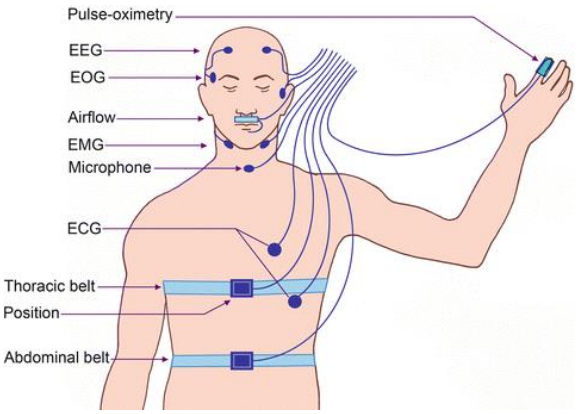

Figure 5. Schematic illustration of instruments used to perform a polysomnography. Figure adapted from Pandi-Perumal et al. Primary Care Sleep Medicine: A Practical Guide. 2014. 29-42.

Polysomnography (PSG) is the combined recording during sleep of several physiological signals. Usually electroencephalogram (EEG), electromyogram (EMG) and electro-oculogram (EOG) are sufficient to discriminate wakefulness from sleep and to distinguish the different sleep stages (N1, 2, 3 and REM) from each other. Other apparatuses such as a pulse-oximetry to measure oxygen levels in the blood can be included if sleep disturbances like *obstructive sleep apnoea* (OSA) are suspected[35].

Although a valuable tool, PSG is expensive to perform, as trained staff is required to apply the equipment. Moreover, the amount of data produced is relatively

[III] **Variable Number Tandem Repeat**; "a tandem repeat from a single genetic locus in which the number of repeated DNA segments varies from individual to individual and is used for identification purposes (as in DNA fingerprinting)"32. In. *Merriam-Webster.com*: Merriam-Webster Inc.; 2011.

cumbersome to analyse and manual analysis is still the most reliable approach. Another issue of PSG is that the wired electrodes attached to the scalp and face may create discomfort and interfere with sleep creating the so called "first night effect". For this reason, several nights of recordings are necessary to obtain reliable data[36]. In clinical practice, PSG is mainly performed to evaluate the presence of sleep-related breathing disorders, periodic limb movement disorder while is "not required nor recommended" to diagnose insomnia[37].

1.1.3.2 Sleep diary

A sleep diary consists in annotating sleep related information, every day for several days. Even if some attempt have been made to implement the use of a Consensus Sleep Diary[38], to this date many different versions exists and are routinely used in clinical practice, but also in research settings. The diary invites the user to report, among others, entries that covers the three nocturnal symptoms of insomnia: sleep onset-time, number of awakenings and time of the final awakening.

The diary is a cheap and accessible tool to register sleep information in a subjective way. For this reason, the sleep-diary is a suitable tool to evaluate the presence of insomnia. This tool is indeed part of the Cognitive Behavioural Therapy for insomnia (CBT-I) as it allows to measure sleep efficiency before and after the intervention[39]. Although useful, sleep misperception and recall bias can affect the quality of the information collected with this method[40].

1.1.3.3 Actigraphy

In order to lessen the subjectivity of the sleep diary, researchers often turn to actigraphy[40], which measures rest and activity through a bracelet containing an accelerometer. Nowadays, activity armbands and activity watches are relatively cheap and therefore can be used in large samples. Unfortunately, this tool is not accurate in insomnia patients since data obtained with this method can be misinterpreted (i.e. the subject may be laying immobile but awake)[40].

1.1.3.4 Sleep questionnaires

There are several hundred questionnaires designed to gather quantitative (sleep time, onset, number of awakenings) and qualitative (sleep quality and depth) information about sleep and evaluate the presence of sleep disturbances[41]. Questionnaires provide subjective reports of these disturbances which can be a limitation for organic sleep conditions, but many are validated in large populations[41]. For research purposes, especially in epidemiological studies with large samples, these are valid, less expensive alternatives to structured and semi-structured interviews, which require more time and need to be administered by professionals[42].

Some of the most commonly used are the Pittsburgh Sleep Quality Index (PSQI)[43], the Insomnia Severity Index (ISI)[42], the Brief Insomnia Questionnaire (BIQ)[44] and the Karolinska Sleep Questionnaire (KSQ)[45]. These four examples ask the respondents to report the presence of symptoms in terms of either frequency (PSQI, BIQ and KSQ) or severity (ISI) [44]. PSQI, however, was not designed to investigate one specific sleep disorder and therefore is not tailored to diagnostic criteria[42].

1.1.4 Pathologies of sleep

Most people would be surprised to know that they lay paralyzed most of the nights during REM sleep. On the other hand, some are very aware of this phenomenon. One peculiar and terrorizing occurring is the so-called *sleep paralysis*, when the dreamer reports to be awake, and conscious but totally paralyzed except for the eyes. This is caused by a lack of synchronization among areas of the brain, where the consciousness centres are "awake" but the motoric areas are still "sleeping"[46].

Even if distressful, the faulty activation of brain areas during sleep paralysis is not harmful. However, sleep can be disrupted by many conditions with more severe consequences. The symptoms of these conditions disrupt sleep in different ways, often overlapping with the symptoms of insomnia.

In the past 10 years, there has been an increased interest towards disordered breathing during sleep and in particular sleep apnoea (SA). SA is characterized by interruption in breathing during sleep for periods long enough to affect oxygen levels in the blood. Snoring often accompanies the "obstructive" type of this condition (OSA). OSA is the most common type of sleep apnoea and it is caused by pharyngeal tissue collapsing when laying supine. In very few cases, SA is classified as "central" (CSA) as the interrupted breathing is caused by a lack of autonomic drive from the brain[47]. In the most severe cases, the person wakes several times during the night with the impression of chocking or drowning. Consequently, patients report poor sleep and/or tiredness during the day[48].

Another condition that may disrupt sleep is Restless Leg Syndrome (RLS). A crawling sensation in the lower limbs when in a resting state cause the patients to feel the impulse to move them in order to feel relief. The sensation often interferes with sleep onset, but awakenings during the night are also common[49]. Psychological hyperarousal is also a common feature for this syndrome; patients report few hours of sleep at night but not necessarily sleepiness during the day. The condition seems to have a complex aetiology, involving low iron levels in the brain. Lack of this element deregulates dopamine, glutamate and adenosine levels producing increased action of these neurotransmitters along the cortico-striatal-thalamic-cortical circuit and the mentioned symptoms[49].

While SA and RLS interfere with sleep quality and quantity, a class of disorders called *circadian rhythm disorders* affect the intrinsic timing of sleep. In *delayed sleep phase syndrome*, affected people have a shifted internal timing and therefore they are not able to fall asleep until the early morning. This condition may be mistaken as a severe case of insomnia, but the patients usually are able to sleep considerably long throughout the day. Even if these are rare and extreme cases, it is possible that the disease is in fact a spectrum, with the less severe cases being diagnosed as insomnia[50].

The mentioned conditions interfere with sleep, but individuals free from any physical or mental condition may still present with problems in sleeping. As the other sleep disorders, insomnia is independent condition.

1.2 Insomnia

Since the 17th century the word *insomnia*, from the Latin negative suffix *in-*, and *somnus,* sleep, has been commonly used for referring to the inability to sleep. As explained in the previous chapter, sleep is an extremely important physiological process and its lack is often underestimated. One night of "bad sleep" and a consequently grumpy day can be rapidly forgotten, but recurrent sleeplessness is a major distress for those who are affected. Moreover, insomnia seems to increase the risk for other disorders and mortality[51-55], producing an enormous socio-economic burden from both direct and indirect consequences[44,56-58].

Insomnia is the most common sleep disorder and comprises of both nocturnal and daytime symptoms. In particular, nocturnal symptoms involve difficulties in falling asleep or maintain sleep. Maintenance problems are in some definitions of insomnia divided between several awakenings during the night and early morning awakenings. Generally, one symptom dominate over the others, but presenting more than one symptoms is not uncommon [59].

The pattern of these nocturnal symptoms of insomnia differs from one person to another and all combinations are possible[60]. Additionally, in the literature, symptoms of insomnia are reported to differ in prevalence, risk of developing a severe mental disorder[61], in electroencephalographic recordings[62] and neuroimaging parameters[63]. Nevertheless, these findings have been overlooked by researchers, leaving the presence of peculiar characteristics of the symptoms of insomnia an open matter. Combinations of different symptoms are taken into account even less, despite the fact that combinations of symptoms more often become chronic than single symptoms[59].

Another aspect that appear to be overlooked is that the nocturnal symptoms of insomnia coincide with different phases of sleep, onset, maintenance and

termination, which are controlled by different molecular processes and neural pathways. Hypothetically, faults in one of these mechanisms could account for the manifestation of one symptom rather than another. Moreover, as sleep is a circadian process that occurs according to an intrinsic schedule, diverse interferences could account for delays or advances of phase producing respectively difficulties in sleep onset and early morning awakenings.

In this chapter, I will report several aspects of insomnia, focusing on individual symptoms, when the literature allows it.

1.2.1 Clinical and research definitions of insomnia

Over the years, research in sleep and insomnia has improved the characterization of this sleep disorder. However, the presence of several diagnostic manuals for psychiatric conditions has created different definitions that are reflected in research settings. On top of this, research-oriented definitions have also been proposed.

Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-5) lists the presence of dissatisfaction with quantity or quality of sleep with at least one of three nocturnal symptoms of insomnia (difficulty initiating sleep, maintaining sleep and early morning awakening) despite the opportunity for sleep. This last criterion is one of the differences with the previous version of the DSM, the DSM-4. In the newer version, a frequency requirement of at least three nights a week was also added and the endurance criterion for the condition was increased from one to three months. The presence of clinically relevant interference with any area of daily functioning (sleepiness, difficulties in focusing, reduced cognitive functioning, etc.) is also given focus. Finally, it should not be possible to explain the disturbance by the presence of other physical or psychiatric condition or use of interfering substances[2].

International Classification of Diseases-10 (ICD-10)[64]: includes the presence of one nocturnal symptom of insomnia among sleep onset and maintenance problems, or non-restorative sleep, occurring at least three times a week for at least one month,

resulting in distress and reduced daily functioning, in the absence of other medical or external factors. Preoccupation about sleeplessness is also listed among the criteria. In respect to the DSM-5 this tool recognizes only two symptoms of insomnia and the required presence of the symptoms is shorter (one month vs three months).

The list of criteria for "chronic insomnia" presented in the *International Classification of Sleep Disorders (ICSD-3)*[65] *are identical to those presented in the DSM-5 and partially the ICD-10* sleep onset or maintenance problems, despite having the opportunity to rest, with consequent impairment at daytime. The disturbance must occur at least three times a week and for at least three months. The third edition of the classification discontinued previous sub-types of insomnia such as psychological, paradoxical and idiopathic, primary as opposed to secondary and comorbid. Unreliability is the reason for omitting these sub-classification of insomnia[65].

Criteria by the American Academy of Sleep Medicine (AASM) [66] give an operational definition of insomnia based on quantitative measure of several sleep parameters. According to these guidelines insomnia is present if sleep onset latency (SOL) exceed 30 minutes, wakefulness after sleep onset (WASO) intervals last more than 30 minutes, sleep efficiency (i.e. the ratio of time spent sleeping over time spent in bed) is less than 85%, and/or total sleep time is less than six and a half hours. As the ICD-10, these criteria also do not include early morning awakenings among the nocturnal symptoms of insomnia.

1.2.2 Epidemiology of insomnia

1.2.2.1 Prevalence

As the definition of insomnia varies, estimates of the prevalence of insomnia in the literature are also very diverse. In population studies, the prevalence of insomnia ranges from 4% using ICD-10 , 15% using research criteria[51,67], 16-22% using the DSM-4 criteria[67,68], and up to 48% when less stringent definition are used [51]. In this last case, the presence of only one insomnia symptom is often used

as a criterion. Refining the definition by including information such as the frequency or severity of symptoms decreases the prevalence by up to 27 points percentage[51]. Finally, estimates from some studies where both diagnostic and quantitative research data are employed report a prevalence of about 15.9%[51].

In Norway, prevalence based on DSM-4 increased from 11.4%[69] in early 2000s to 15.9% after 10 years. Stratified analysis by symptoms showed a statistically significant increase only for the prevalence of sleep onset insomnia (from 13.1% to 15.2%) [68].However, the prevalence was reduced to 7.9% when DSM-5 criteria was used[70].

When it comes to individual nocturnal symptoms of insomnia, maintenance insomnia is the most frequently reported symptom in many, if not all, studies, being present in up to 70% of people reporting insomnia[61,71,72].

1.2.2.2 Socio-economic burden of insomnia

If we accept the mentioned estimates for the prevalence of insomnia as valid, 118 million persons in Europe are potentially affected by insomnia, over 1 billion in the whole world. Both indirect costs such as lost productivity and direct costs such as medical consultations, medications and therapeutic interventions for these numbers are enormous. One study on the USA population, where people suffering with insomnia are 50-70 millions, reported 63 billion dollars in annual costs[73].

The major inflating agent of the cost of insomnia appear to be lost productivity[58,73]. Estimates from the Canadian province of Quebec (population 5.6 millions) showed that annual indirect costs such lost productivity amount to 5 billion dollars alone on a total cost of insomnia of 6.6 billions [56]. This factor constituted the 76% of all the costs associated with insomnia. Eliminating insomnia is estimated to decrease the whole work lost productivity by 5.4% to 7.8%[73]. A Norwegian study reported that insomnia had a higher impact on work disability (6.7%) than depression alone (3.8%) and combined insomnia and depression (5.3%) [57].

For the direct costs, medical consultation are estimated to account for 33% of the burden while purchasing of alcohol as an aid to induce sleep to a much higher

58%[56]. This last factor is an indicator of collateral deleterious factors emerging from insomnia that might have been overlooked in the estimates of the economic burden.

1.2.2.3 Demographic factors influencing the prevalence of insomnia

Demographic factors such as age and sex, that often influence disease prevalence, also have a role in insomnia. In respect to age, sleep length decreases until age 60 settling to an average of 7.5 hours per night in healthy elderly. Sleep architecture also changes with age and time spent in SWS and REM sleep is reduced[74]. All these elements are considered physiological changes of aging: indeed healthy elderly seldom report sleep complaints[74]. Still, epidemiological studies have shown a 10% increase of the probability of insomnia for each age decade. It is likely that a large number of somatic and psychiatric conditions common in the geriatric population work as mediators in the relationship between aging and insomnia[51].

As to differences between sexes, women have a Risk Ratio (RR) of 1.4 compared to men to develop insomnia. This means that being female gives a higher probability of developing insomnia by 40%[75]. Hormonal fluctuations are seen as the possible culprit of these differences as women often report insomnia in conjunction with menstruation, pregnancy and menopause. Oestrogen and progesterone receptors are expressed in the basal forebrain in several areas involved with sleep regulation and oestradiol have been shown to inhibit the sleep promoting action of the ventrolateral preoptic area (VLPO) [76]. It appears that the difference in prevalence between sexes appears after onset of menarche. Interestingly, it is also at this age that the odd-ratios for depression becomes unfavourable for girls[77].

1.2.3 Comorbid insomnia

Between 70% and 90% of cases of insomnia can be classified as *secondary insomnia* or, according to the current terminology, *comorbid insomnia*. The change in terms was dictated by an increased awareness that insomnia appearing in the presence of another condition often becomes chronic and does not ameliorate by treating the primary disorder[51].

Among the somatic complaints, pain is one of the most obvious causes of disrupted sleep, increasing by more than 3 times the chances of developing insomnia[52]. However, insomnia is frequent in individuals affected by several other conditions such as cancer (OR 2.6), cardiovascular diseases (OR 2.3) and neurological problems (OR 4.6) to cite only a few[52].

Chronic psychiatric disorders also have a prominent comorbidity rate with insomnia like manic, mood and anxiety affections, such as schizophrenia[53], bipolar disorder (OR 3.7) [54,55], Major Depressive Disorder (MDD, 5.8), Generalized Anxiety Disorder (GAD, OR 5.6) and Post-Traumatic Stress Disorder (PTSD, OR 4.9)[55].

Comorbidity of insomnia and psychiatric disorders may also vary according to experienced nocturnal symptoms. A Swedish study evaluating the chance of being awarded a disability pension among individuals presenting with insomnia, reported different hazard ratio for the different symptoms of insomnia. In particular, hazard ratio for receiving the disability pension due to a chronic mental disorder was 1.5 for men reporting maintenance insomnia and double for sleep-onset or early morning awakenings. For women the highest HR was for early morning awakenings (2.1) followed by maintenance insomnia[61].

1.2.4 Explanatory models of insomnia

Through the years, several attempts to model the development and establishment of chronic insomnia have been made[29]. These models do not exclude each other but attempts to explain the complexity of insomnia from the point of view of different research areas.

Generally, insomnia can be explained by the *Diathesis-stress* model[78]. This model is also called 3-P because it suggests that insomnia rise from the interplay of *predisposing, precipitating* and *perpetuating* factors. Genetic, physiological or psychological traits which are intrinsic in an individual may interact with causal triggering factors. These may also be psychological and physiological, but environmental disturbances are also to be considered. Finally, behavioural, psychological, environmental and physiological elements can perpetuate

unhealthy sleep habits. However, this model can be considered true for many complex diseases. Other models have been developed with more specificity to the characteristics of insomnia.

One of the first psychological models for insomnia, the *Stimulus control model*[79], was proposed by Bootzin in the 1970s. This model is based on well-known phenomena: classical conditioning. In the famous experiment by Ivan Pavlov a bell (stimulus) was rang every time food was presented to a dog. After some time exposed to the stimulus, the dog would salivate every time it heard the bell. Similarly, insomnia is developed by associating the bedroom to wakefulness, in a perpetuating chain-loop. The model, even if largely heuristic, is considered valid and is the base of the *stimulus control therapy*. The goal of this behavioural therapy is to break the vicious connection between the bed and wakefulness by going to bed only when the sleep pressure is so elevated that sleep will be inevitable.

Psychological factors and in particular psychological arousal are the base of two other models, the *Cognitive* model[80] and the *Psychobiological inhibition* model[81]. In the first, paradoxically, worry about losing sleep and being tired may cause anxiety that inhibits sleep. Similarly, in the second, a stress state lead the individual to focus toward stress cues, setting the brain in a state of alertness that impedes sleep[82].

The more recent models are brought forward from the neurobiological side of the matter. The *Neurocognitive model*[83] uses biological evidence that could explain elements from the *Stimulus control model* and the psychological models. The neurobiological models attempt to explain findings of polysomnographic studies that found a great mismatch between objective measurements and subjective reports of sleep. In particular, individuals with insomnia report a long time to fall asleep and awakenings that often is not revealed by the instruments. Several hypothesis to explain this misperception have been proposed but only few have evidence in their support[84]. One of them is sleep state misconception: people affected by insomnia have a tendency to misperceive sleep state as wakefulness[84].

Electroencephalogram records of individuals with insomnia reveal the peculiarity of high cortical activity around sleep time and during NREM sleep[85,86]. This type of activity is normal during wakefulness and REM sleep as the cortex is engaged in sensory processing, memory formation and consciousness[87]. Activation of specific neuronal circuits during the first stages of sleep or the other NREM intervals may be perceived as wakefulness, even if the rest of the brain is engaged in sleep[29]. This state of cortical hyperarousal is possibly the product of underlying psychological distress[84].

The theory of hyperarousal is supported also by studies showing a deregulation in homeostatic sleep pressure in people with insomnia. They generally lament feeling tired during the day, but they do not present sleep rebound mechanism as normal sleepers. Markers of sleep rebound such as increase in SWS and shortened sleep onset latency (during the day or following a night total sleep deprivation) are reduced in people with insomnia[88].

Another element that supports the link between insomnia and sleep misperception is brief awakenings. Cyclic alternating patterns (CAP) are EEG anomalies that indicate sleep instability[89]. These are much more common in individuals with insomnia[84]. Actual fragmentation of sleep in short bouts during the phase N1 is also perceived as wakefulness in individuals with insomnia[90].

In conclusion, the models summarized here present a complicated framework where neurophysiological elements (i.e.: hyperarousal, misconception, false believes, learned cues, external and predisposing factors) are mixed at different levels. None of the models can be considered inexact nor the ultimate one, as all consider different perspectives of the complex disease of insomnia.

1.2.5 Predisposing, precipitating and perpetuating factors of insomnia

As presented in the 3-P model, insomnia is caused by predisposing, precipitating and perpetuating factors. The same factor can belong to more than one of these three categories creating many different anamneses for each individual case of insomnia. Psychological distress and physical conditions are the most common

predisposing factors, but they can also work as precipitating agents together with environmental factors (noise, blue light from screens etc.). Maladaptive lifestyle choices (irregular sleep schedule, evening caffeine intake etc.) and beliefs about sleep (i.e. misconception about one's sleep and insomnia) are common perpetuating elements that increase the risk of chronicity [91,92].

This thesis focuses on genetic factors that may give predisposition to insomnia and psychological factors that can have both a predisposing and precipitating role.

1.2.5.1 Genetics of insomnia

From experience, the idea that insomnia has a genetic component takes many people by surprise. Still, as virtually all physiological traits differ among individuals due to their different genetic profiles, also parameters relative to sleep are subject to this variability.

Heritability[IV] (h^2) of insomnia is moderate but confirmed by numerous twin studies. Depending on the definition of insomnia used and its sub-phenotypes h^2 ranges between 0.22 to 0.59[93]. This means that genetic variability plays a moderate role in the insurgence of insomnia. For this reason, insomnia can be categorized as a *complex disease:* the result of independently occurring predisposing Genetic profiles and incidental Environmental factors, which trigger the disease (G×E).

Attempts to explain the genetic side of insomnia were conducted since the early years 2000s by hypothesis driven studies on specific candidate genes first, and then by hypothesis-free Genome Wide Association Studies [V](GWAS)[94].

[IV] **Heritability**: *"[…]the proportion of observed variation in a particular trait (such as height) that can be attributed to inherited genetic factors in contrast to environmental ones"* (n. 2 d.)32. Ibid.

[V] **Genome Wide Association Studies:** methodology consisting of testing several thousand genetic variations across the genome for association with a trait in order to identify genes that influence that trait[101].

Candidate gene studies

The quest for candidate genes for insomnia started with the *GABA* (γ-aminobutyric acid) receptors[95]. Then, observations of sleep disruption in animals with silenced circadian genes brought these genes in the spotlight[96]. Metabolic pathways for neurotransmitters followed right after with the dopaminergic[97] and serotonergic systems[98] among the most investigated. Finally, molecules with known ancillary roles in sleep like adenosine[99], orexin/hypocretin[100] and melatonin[101] caught the attention of researchers.

GABA is the primary inhibitory neurotransmitter in the brain. Its role in sleep was hypothesized first in the 1960s and several later studies have confirmed it[95,102]. GABA receptors are also the target of hypnotics and anxiolytic drugs. Surprisingly, very few genetic studies have investigated the variability of the GABA pathway in relation to insomnia[103]. After a study identifying a functional missense mutation[VI] in the $GABA_A$ receptor in one patient reporting insomnia[95], to date there is no study actively investigating genetic association of the GABAergic system and insomnia.

Given the high rate of comorbidity of insomnia with psychiatric disorders, genetic predisposition of this sleep disorder has often been investigated using samples of psychiatric patients. The design of these studies makes it difficult to generalize the findings to individuals with insomnia without other psychiatric diagnosis. However, genetic predisposition for insomnia may derive mostly from genes involved in responsiveness to stress rather than sleep physiology[104]. Evidence suggest that insomnia and Generalized Anxiety Disorder (GAD) are, from a genetic point of view, the same disease while Major Depressive Disorder (MDD)

[VI] **Missense mutation**: *"relating to or being a gene mutation involving alteration of one or more codons so that different amino acids are determined"*. For example, a gene is composed by numerous molecules that are called nucleotides A, C, G and T for simplicity. The gene sequence is a "recipe" for a protein. From the beginning of the gene each group of three nucleotides can be considered as the name of an "ingredient" in our "recipe". Changing one single letter in a sequence would therefore give another ingredient, (ATC to ACC, as butter to cutter) or out of the metaphor, another amino acid.

shares between 56% (in females) to 74% (in males) of genetic background with insomnia[105].

Genetic studies exploring this shared genetic profile are scarce and focus almost exclusively on gene *SLC6A4* that codes for the *serotonin* transporter (5-HTT). The serotonergic system in the brain is involved in mood[106] and anxiety disorders and serotonin transporters are targets for several psychiatric drugs, selective serotonin reuptake inhibitors (SSRIs) among others. Serotonin is also involved in a ubiquitous way in wakefulness regulation: both promoting and contrasting it [107].

The promoter region of *SLC6A4* has a notorious polymorphic region named 5-HTTPR that comprises a variable number tandem repeat (VNTR) locus, which includes two Single Nucleotide Polymorphisms[VII] (SNPs). At least 14 allelic[VIII] combinations of the number of repeats and SNPs exist[108]. Most of the studies concentrated on the differences between the long (L) and short (S) variants of the VNTR of the serotonin transporter gene. The short allele in the presence of stressors such as caring for a dement spouse[109] or job-related stress[110] increased the chances of experiencing insomnia. This framework seems to be totally reversed when shift work enters the picture. Changing sleep pattern due to night shifts seems to trigger insomnia in carriers of the L_A-L_A genotype, with A referring to the allele of the SNP rs25531 (A/G). The genotype was associated with increased binding potential[IX] (BP) of the transporter for serotonin[112,113] as a result or together with increased transporter expression[114]. The reasons for these inverted elements being both associated with increased risk for insomnia is difficult to interpret but it might be the product of the differences in interacting factors (psychological stressor versus circadian stressor) and neurotransmitters pathways.

[VII] **Single Nucleotide Polymorphism**: a variation in a single nucleotide of the sequence of DNA that occur with a frequency of at least 1% in a population. Under this limit, the mutation is called *rare variant*.

[VIII] **Allele**: one of the possible forms that a polymorphic locus can assume.

[IX] **Binding potential**: a measure of a receptor activity that express its ability to bind molecules[117].

Another molecule involved in sleep is *adenosine*, a by-product of cellular metabolism. Adenosine works as an inhibiting agent for cholinergic neurons and has a popular antagonist, caffeine[22]. Genetic studies focused on Adenosine deaminase (ADA), an enzyme involved in adenosine breakdown. In animal models, the gene coding for this enzyme was involved in increasing sleep pressure[22] while in humans the majority of studies focused on several SNPs causing severe immunodeficiency[115]. One annotated SNPs with less serious apparent consequences, rs73598374 (G22A, Asp8Asn), causes decreased enzymatic activity in heterozygous carriers (G/A) and apparently may lead to increased time spent in SWS and fewer awakenings during the night. The genotype, that is present in 8-12% of the population, may have a protective effect towards insomnia, at least from its maintenance form[34]. SNP rs12256138 on Nucleoside transporter gene, related to adenosine metabolism, *SLC29A3*, was associated with early morning awakenings in women experiencing depression. Decreased function of the product of *SLC29A3* as adenosine transporter may explain the symptoms in carriers of the SNP[116].

As explained in chapter 1.1 sleep timing is regulated by an intrinsic molecular clock. Genes that code for the gears of this timekeeper are defined circadian. Only a few studies have considered these genes as possible candidates in the susceptibility for insomnia. Moreover, the results are sometimes contradictory and self-limited.

Circadian genes were first studied in animal models. Knockout (KO) mice (i.e. with a silenced or depleted gene) for circadian genes revealed altered sleep-wake cycles and sleep architecture. KO mice for gene Bmal1, equivalent to human *ARNTL1*, showed an attenuated circadian rhythm and more fragmented sleep, but overall longer sleep-periods[117]. In humans, SNP 3111T/C on gene *CLOCK* (Circadian Locomotor Output Cycles Kaput) was associated with decreased sleep need and nocturnal symptoms of insomnia in patients presenting bipolar disorder[96] as well. BP is also independently associated with several polymorphism in circadian genes, and it seems possible that people with this disorder have a weakly entrained

circadian clock[118,119]. Conversely, SNP 3111T/C did not influence the presence of insomnia in patients diagnosed with MMD[96,120].

PER (Period) genes are also reported in association with insomnia but the interaction seems to be mediated by psychological distress. Polymorphisms in both *PER2* and *PER3* were associated with insomnia in samples of stressed workers[121] and people affected by alcoholism[122].

Genome Wide Association Studies

The development of low-cost technologies for genomic analyses made possible what appeared as laying out every straw in the haystack to find the long-missed needle. Genome Wide Association Studies (GWAS) allowed to have an overview of several thousand SNPs and get an overview of those statistically more common in individuals affected by a condition or presenting a trait (phenotype). However, also this approach produced fragmented results for many traits, insomnia included. Nevertheless, this approach allowed genetic research on insomnia to take new ways, even if the paucity of follow-up studies makes the findings often self-limited.

The first GWAS on insomnia is one exception where the main study was followed by experimental set ups to validate the findings[123]. Variations in genes *ROR1* and *PLCB1,* previously associated respectively with BPD[124] and animal models of schizophrenia[125], found to be linked to insomnia. These genes are involved in relatively unspecific cellular signalling mechanisms and therefore it was not surprising that genetic variation in their sequence were associated to different syndromes. Another gene*, CACNA1A* (calcium voltage-gated channel subunit alpha1 A), involved in synaptic function and plasticity, was among the significant signals. Interestingly, a following GWAS on insomnia and sleep traits reports among their higher hits a gene for another subunit of calcium channels, *CACNA1C* [126]. Even if not reaching GWAS significance level *($\alpha=10^{-8}$)*, the signal coming from this gene created a continuum between these two studies. Yet, these genes code for proteins that interacts with numerous other proteins to accomplish several molecular functions. It is possible that variations in their sequences give

predisposition for different neuropsychiatric disturbances while epistatic[X] interactions, epigenetics[XI] and environment factors define the final phenotype.

The ability of GWAS of revealing overlapping traits was also clear in the case of gene *MEIS1*. First associated with Restless Leg Syndrome (RLS), *MEIS1* gave the strongest signal also in two studies on the same sample but different operationalization of the disorder [127,128]. RLS manifest itself as nocturnal disturbances as insomnia does, which makes researchers suspect that the two conditions are interconnected: RLS symptoms could be the cause of disrupt sleep or insomnia could be an early symptom of RLS. However, a third study introduced another possibility. SNPs in *MEIS1* were more frequent in patients diagnosed with both insomnia and RLS but not in those with only insomnia[129]. Unfortunately, the study did not have enough power to prove ultimately this difference, but it gave a warning about phenotyping precision in genetic studies in large populations. In other words, the connection between RLS and insomnia seems to be a case of unspecific phenotyping more than one of pleiotropy[XII].

Conversely, pleiotropy between insomnia and several sleep traits seems more likely. Traits like sleep length, chronotype and sleep latency are potential proxies for predisposition to developing insomnia[130]. On these bases, results from GWAS on sleep traits produced many results that could hide cases of pleiotropy with insomnia.

Allebrandt et al. investigated sleep duration in a large population sample, identifying rs11046205 in gene *ABCC9* (ATP-binding cassette, sub-family C, member 9) coding for an ATP-sensitive potassium channel (K_{ATP} channel) subunit. The finding was reproduced in an independent sample. Moreover, it was observed

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[X] **Epistasis**: *"Suppression or inhibition of the phenotypic expression of a gene by a second, non-allelic gene (i.e. a gene at a different locus); (also more generally) interaction between non-allelic genes. Also: an instance of such suppression or interaction". (n.3 d.) 19. In. Oxford English Dictionary: Oxford University Press; 2019.*

[XI] **Epigenetics:** *"the study of heritable changes in gene function that do not involve changes in DNA sequence"32. In. Merriam-Webster.com: Merriam-Webster Inc.; 2011.*

[XII] **Pleiotropy:** *"the phenomenon of a single gene influencing two or more distinct phenotypic traits". 32. Ibid.*

that *Drosophila Melanogaster* (fruit fly) with silenced *ABCC9* homologous gene slept later than wild type flies at night-time[131]. K_{ATP} channels regulate sleeps through their role in safeguard of the brain during energy shortage. When blood glucose is low these K_{ATP} depolarize neurons in several groups of neurons. The wakefulness-inducing role of these neurons is contrasted and sleep can prevail[132].

Sleep phenotypes were investigated in several other GWASs. Usual bedtime was associated with gene *CSNK2A2.* Its homologous is a recognized component of the circadian machinery in *D. Melanogaster* (fruit fly) while in humans was previously associated with Familial Advanced Sleep Phase Syndrome. Sleep duration was associated with *PROK2*, another gene associated with circadian rhythmicity [133]. Another, larger study on sleep duration brought attention to a gene involved in thyroidal function, *PAX8* [134]. In this case, lack of follow up studies leaves explanation of these findings largely speculative if not totally lacking.

One case where results needed less speculative effort was that of a GWAS on the trait "being a morning person" or "morningness". Results included highly significant *p*-values for seven SNPs on well-known circadian genes and for four on genes with a "plausible circadian role"[135].

1.2.6 Insomnia, anxiety and depression: comorbid or causative interaction?

Insomnia is a frequent comorbidity also of psychiatric conditions such as depression[136] and anxiety disorders [137]. Insomnia increases the risk of depression by two to three times[136,138], while people with depression are three times more likely to experience insomnia[72]. Diagnosis of an anxiety disorder poses a four time higher risk of insomnia, whereas insomnia increase by three times the chances of developing anxiety[72,138].

Insomnia may emerge before, after or concomitantly with depression or anxiety, however some scenarios appear to be more frequent than others. More often, anxiety precedes insomnia, while depression arises after experiencing insomnia[139,140] and there is evidence for these to be the only significant predictive

patterns[139]. It is clear that these conditions are interconnected but the nature of this correlation remains unsolved and may be diverse.

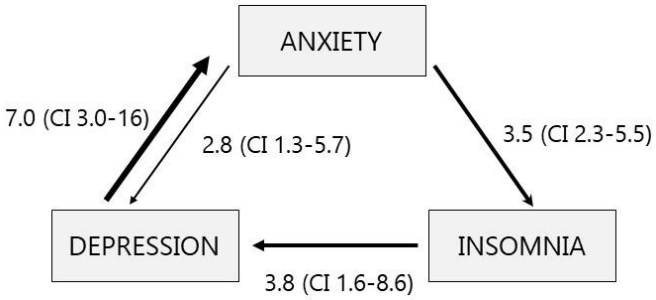

Figure 3. Relationship among insomnia, depression and anxiety with HR according to Johnson et al. J Psychiatr Res 2006;40(8):700-708. (Figure adapted from Glidewell et al. Sleep Med Clin. 2015;10(1):93-99.)

This model seems to be supported by studies evaluating the effect of therapeutic interventions designed for one condition, in cases presenting insomnia with anxiety or depression. In example, mono-therapeutic Cognitive Behavioural Therapy (CBT) designed to treat insomnia (CBT-I) have a limited effect on anxiety[141] while CBT for anxiety is beneficial to both conditions, seemingly by reducing *anxious arousal* (i.e. somatic symptoms of anxiety)[142]. This agrees with the evidence that alleviating anxiety and specifically somatic tension can ease sleep[143]. On the other hand, CBT-I is more beneficial than CBT for depression in cases of comorbid depression and insomnia[144].

Uhde and colleagues formulated two theoretical models to explain the etiological interaction of anxiety and insomnia. This can also be adapted to the relationship between insomnia and depression.

> "*The first suggests that anxiety and insomnia represent different dimensions of a single dynamic neurobiological diathesis (i.e., the same abnormality with a spectrum of symptoms)".*
>
> *In the second model, anxiety and insomnia may represent different neurobiological disorders. In this model, two circumstances may explain the high comorbid association: 1) anxiety and insomnia are separate disorders,*

each of which separately causes similar or overlapping downstream complications; or 2) anxiety and insomnia are both critically influenced by another widely prevalent third factor." [145]

The authors compare model 2.1 to the same relationship between hypertension and renal disease: two separate conditions that lead to one another if left untreated. Model 2.2 is effectively explained using another medical equivalent: the role of hypercholesterolemia in aggravating diabetes and cardiovascular disease.

Comorbidity between depression and insomnia seems to have some symptom specific aspects. A piece of knowledge recalled by many is the relationship between early morning awakenings and depression. Surprisingly, evidence supporting this information is lacking. In fact, early morning awakening alone is reported in the DSM-5 as a specific symptom of *melancholic depression*. As I could not find evidence in the literature supporting this connection, I suppose that the notion comes from clinical practice. However, some studies conducted after the DSM-5 was released, seems to support the specificity of the association. Early morning awakening is the only symptom associated with thinned grey matter of the orbitofrontal cortex (OFC)[146]. Interestingly, reduced grey matter in this area is also reported in individuals affected by insomnia with comorbid depression[63]. A result of a compensatory mechanism, this comorbid state is also characterized by increased connectivity of the OFC with subcortical regions[147].

1.2.7 Nocturnal symptoms of insomnia as separate entities

In the course of the previous chapters, I presented several aspects of sleep and insomnia in order to give an idea of the complex landscape in which the research question of this thesis arises. Neurophysiological studies of sleep show that this fundamental phenomenon has a complex regulatory system and architecture. The multiplicity of sleep stages and the corresponding molecular mechanisms give the potential for different characteristics of symptoms of insomnia.

Yet, as mentioned before, the three nocturnal symptoms of insomnia have seldom been investigated for their different characteristics and they are considered

equivalent in clinical and research definitions of insomnia. From an epidemiological perspective the symptoms differ from each other in simple aspects such as prevalence[59,61,62,148]. Maintenance insomnia seem to be always the most reported symptom, but its high prevalence seems not to be reflected in its implications. This symptom give the lowest risk to develop invalidating mental conditions[61] and was not associated to higher levels of anxiety or depression[59,62,148]

In contrast, sleep onset insomnia was associated with increased risk of mortality in Norwegian and Finnish men, but not in women[149]. Moreover, in men, sleep onset and terminal insomnia triplicated the chances of developing a debilitating mental condition, while maintenance insomnia increases the risk by a much lower 50%. In women, the pattern was different, with only terminal insomnia having an effect[61]. Neuroimaging studies have also showed different patterns among the symptoms. Reduction in the grey matter OFC was present exclusively in people experiencing terminal insomnia[146]. Finally, in an EEG study, individuals presenting with sleep onset insomnia displayed different spectral characteristics that those with maintenance insomnia. Because of these differences, the authors of the article invite to avoid mixed samples in upcoming EEG studies[62].

These few pieces of evidence suggested that the differences among the symptoms could extend to other characteristics. Genetic background and psychopathological factors are respectively new and classic topics in insomnia research that offer a starting point to continue charting these differences.

2. Aims of the studies

General aim

The general aim of this thesis was to investigate differences in genetic and psychopathological characteristics of the individual nocturnal symptoms of insomnia and their combinations.

Study 1

The aim of this study was to investigate the presence of association among different patterns of symptoms of insomnia and SNPs in circadian genes

Study 2

The aim of this study was to investigate whether SNPs previously reported to be associated with sleep-related phenotypes are associated also with different patterns of symptoms of insomnia.

Study 3

The aim of this study was to report and compare levels of anxiety among individuals reporting different patterns of symptoms of insomnia.

Study 4

The aim of this study was to assess and compare the level of symptoms of depression according to individual nocturnal symptoms of insomnia

3. Materials and methods

3.1 The HUNT study

The studies included in this work were conducted using genetic and health information from participants in the Nord-Trøndelag Health Study (the HUNT study, Norway). This cohort study started in 1984 inviting all citizens aged 20 or older of the Norwegian region of Nord-Trøndelag to contribute with both biological material and extended health information to the study. For the studies presented here, we selected individuals from the third round of the HUNT study (HUNT3, 2006-08).

HUNT3 participants donated urine, blood and saliva (Young-HUNT3) samples that were used to extract biochemical data and DNA. The samples are stored in the HUNT Biobank (Levanger, Nord-Trødelag, Norway), a state-of-the-art infrastructure provided of automatized handling technology that can stock several million matrix tubes.

3.2 Symptoms of insomnia

The three nocturnal symptoms of insomnia were determined by using the answers to three questions contained in the HUNT3 Questionnaire 2 (Sleep section)[150]. The questions express the first criterion for the diagnosis of insomnia as stated in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) [2].

How often in the last 3 months have you:

Had difficulty falling asleep at night? (Sleep onset insomnia)

Woken up repeatedly during the night? (Maintenance insomnia)

Woken too early and couldn't get back to sleep? (Terminal insomnia)

Possible response options were: "Never/seldom", "Sometimes", "Several times a week".

In all four studies, individuals who answered "Several times a week" to at least one question were classified as cases, while those who answered "Never/seldom" to all three questions were classified as controls. Cases were further divided in seven subgroups depending on the pattern of reported symptoms.

3.3 Genetic studies

The genetic data used in study I and II were obtained by the HUNT databank. The data were produced following standard procedures of quality controls.

The software PLINK was used to select SNPs that had Minor Allele Frequency [XIII](MAF) above 5% and those that did not respected the Hardy-Weinberg equilibrium[XIV] (p-value<0.05).

FastIndep[151] was used to identify and exclude individuals presenting relatedness up to the third degree (kinship coefficient ≥ 0.0884)

The studies included 6029 individuals, 3602 cases and 2427 controls.

3.3.1 Study 1

We selected 81 tag SNPs in nine circadian genes: *PER1, PER2, PER3, CRY1, CRY2, TIMELESS, CLOCK, ARNTL and REV-ERBα*. The application Tagger included in the software Haploview was used to select the SNPs with a buffer region of 2kb around each gene. Function of the SNPs was predicted using SNPinfo Web Server[152].

3.3.2 Study 2

This study included 67 SNPs previously reported by five GWAS. Selected the SNPs by searching the GWAS catalog[153] with the keywords "insomnia", "sleep" and "chronotype" and selected 52 SNPs reported in four studies published before 2016

[XIII] **Minor Allele:** the allele of SNP that is less present in a population.
[XIV] **Hardy -Weinberg equilibrium:** theoretical concept that model the frequency of the possible genotypes (AA, AB, BB) that can be found at a genetic locus in an ideal population were the influences on the genetic pool are absent (absence of immigration, random mating etc.).

[126,133,134,154]. Results of another relevant study on "morningness", consisting of 15 SNPs were included afterwards [135]. 9 SNPs did not pass quality controls.

3.3.3 Statistical analyses for study 1 and 2

We used multinomial logistic with pattern of symptoms of insomnia and controls as a categorical dependent variable (DV) and the SNPs as independent variables (IV) one at the time. The results were corrected Benjamini-Hochberg False Discovery Rate (FDR). The analyses were conducted using RStudio (Version 1.0.136).

3.4 Studies on psychopathological traits

Study III and IV investigated differences in psychopathological traits as measured by the Hospital Anxiety and Depression Scale (HADS)[155]. The HADS was designed as a self-assessment tool for hospitalized patients to measure levels of anxiety and depression. The questionnaire includes two subscales, one for anxiety and one for depression, which can produce partial results. These values can be summed to produce a total score. Results can be used as a continuous scale, but four grades of increasing severity have been defined: Normal (0-7), Mild (8-10), Moderate (11-14) e Severe (15-21).

Studies 3 and 4 included 7933 individuals, 4317 cases and 3616 controls.

3.4.1 Study 3

In this study we used results of HADS-A as a measure of anxiety and compared mean scores among the pattern of symptoms of insomnia.

3.4.2 Study 4

In the fourth study, we analysed differences in mean scores for the depression subscale of HADS (HADS-D) among patterns of the symptoms of insomnia.

3.4.3 Statistical analyses for study 3 and 4

We used linear regression with the chosen HADS subscale as dependent variable and pattern of sleep symptoms as a seven categories independent variable. The regression analysis was adjusted for sex and age. Pairwise comparisons among all patterns of symptoms were conducted and p-values corrected using Bonferroni adjustment accounting for all the pairwise comparisons between the seven groups. Chi-squared test was used to compare proportions of the seven patterns in the four grades of severity for HADS results.

3.5 Ethics

The project under which these studies have been conducted was approved by the Regional Committee for Medical and Health Research Ethics of South-East Norway (reference number 2016/672) on date 04.27.2016. Participants in the HUNT study have signed a written informed consent form through which they allowed the use of their data and biological samples for research purposes. All participants are anonymized and can withdraw their data from the study at any given time.

The genetic variations used in this study are not known to have a direct causative relationship with any known disease.

4. Results

4.1 Review of study 1

Variations in circadian genes and individual nocturnal symptoms of insomnia. The HUNT study.

Bragantini D., Sivertsen B., Gehrman P., Lydersen S., Güzey I.C.

Chronobiology international. 2019; 36(5):681-8.

The aim of this study was to investigate the relationship between SNPs in nine circadian genes and patterns of symptoms of insomnia. We used data from 3602 individuals reporting the three symptoms of insomnia in all their possible combinations and 2427 controls. The 81 SNPs were tested one at the time for differences among seven different patterns of symptoms of insomnia.

RESULTS:

- Twenty-six variations presented promising odds-ratios and significant *p*-values (α=0.05) but none remain significant after FDR correction.
- Before correction, all significant SNPs in *PER3* were associated with reporting all three symptoms at the same time.
- SNPs in *CRY* genes were associated with terminal insomnia alone or in combination with other symptoms.
- Variations in gene *ARNTL* was mostly associated with combination of symptoms that included sleep onset insomnia.

Table 1: The 25 SNPs that presented significant p-values before correction for multiple statistical testing.

SNP	CHR	Gene	Ref. allele	Other allele	MAF	GROUP	B	OR	OR 95% CI	P-value
rs11022761	11	*ARNTL*	T	C	0.09	SOI+TI	-0.95	0.39	[0.15 to 0.97]	0.0417
						TI	-0.26	0.77	[0.59 to 1]	0.0458
rs3816358	11	*ARNTL*	A	C	0.1	SOI+TI	-0.86	0.42	[0.18 to 0.98]	0.0454
						SOI+MI+TI	0.24	1.27	[1 to 1.61]	0.0480
rs7126796	11	*ARNTL*	C	A		SOI+TI	-0.84	0.43	[0.22 to 0.85]	0.0147
						SOI	-0.21	0.81	[0.66 to 0.99]	0.0444
rs12363415	11	*ARNTL*	G	A	0.2	SOI+MI+TI	0.22	1.25	[1.03 to 1.52]	0.0260
rs10861688	12	*CRY1*	T	C	0.2	TI	0.39	1.48	[1.19 to 1.84]	0.0004
						MI+TI	0.24	1.27	[1.03 to 1.57]	0.0269
rs12368868	12	*CRY1*	G	A	0.07	MI+TI	0.32	1.37	[1.03 to 1.83]	0.0300
rs11038698	11	*CRY2*	C	T	0.07	TI	-0.47	0.63	[0.44 to 0.89]	0.0082
rs11038699	11	*CRY2*	G	A	0.29	SOI+TI	-0.52	0.59	[0.37 to 0.96]	0.0352
rs3824872*	11	*CRY2*	A	C	0.21	TI	-0.31	0.73	[0.58 to 0.92]	0.0084
rs7121775*	11	*CRY2*	C	T	0.23	SOI+TI	-0.57	0.57	[0.34 to 0.95]	0.0309
rs2292913	11	*CRY2*	G	A	0.07	SOI+MI+TI	0.30	1.35	[1.03 to 1.77]	0.0312
rs2518023*	17	*PER1*	T	G	0.08	SOI	-0.29	0.75	[0.58 to 0.97]	0.0274
rs2585408*	17	*PER1*	T	C	0.41	MI	-0.18	0.84	[0.72 to 0.98]	0.0228
rs3027160*	17	*PER1*	C	T	0.21	MI	0.15	1.16	[1 to 1.35]	0.0467
rs3027178†	17	*PER1*	G	T	0.28	MI	0.15	1.16	[1 to 1.34]	0.0446
rs4663866*	2	*PER2*	C	A	0.08	MI	0.23	1.26	[1.04 to 1.53]	0.0201
rs934945§	2	*PER2*	T	C	0.21	SOI+MI	-0.23	0.79	[0.63 to 0.99]	0.0373
rs10462018	1	*PER3*	T	C	0.16	SOI+MI+TI	0.23	1.25	[1.02 to 1.54]	0.0304
rs228666	1	*PER3*	C	T	0.34	SOI+MI+TI	-0.20	0.82	[0.68 to 1]	0.0448
rs697690	1	*PER3*	C	T	0.34	SOI+MI+TI	0.23	1.26	[1.04 to 1.54]	0.0199
rs228692	1	*PER3*	A	G	0.06	SOI	0.26	1.29	[1.01 to 1.66]	0.0414
rs875994	1	*PER3*	C	T	0.18	SOI+MI+TI	-0.39	0.68	[0.54 to 0.84]	0.0004
rs2071427	17	*REV-ERBα*	T	C	0.26	SOI+TI	-0.53	0.59	[0.36 to 0.96]	0.0355
rs4795424*	17	*REV-ERBα*	C	A	0.2	MI+TI	-0.26	0.77	[0.62 to 0.96]	0.0184
rs883871	17	*REV-ERBα*	A	G	0.12	SOI+TI	-1.01	0.36	[0.17 to 0.8]	0.0120
rs17441402	12	*TIMELESS*	T	A	0.14	SOI	-0.22	0.80	[0.65 to 1]	0.0457

SOI=Sleep onset insomnia. MI=Maintenance insomnia. TI=Terminal insomnia. ARNTL = Aryl Hydrocarbon Receptor Nuclear Translocator Like. CRY= Cryptochrome Circadian Regulator. REV-ERBα=NR1D1, Nuclear Receptor Subfamily 1 Group D Member 1. PER = Periodic Circadian Regulator. TIMELESS = Timeless Circadian Regulator.

Predicted functions according to SNPinfo Web Server: * =Transcription Factor Binding site. †=Splicing site. §= Missense mutation.

4.2 Review of study 2

Genetic polymorphisms associated with sleep-related phenotypes; relationships with individual nocturnal symptoms of insomnia in the HUNT study.

Bragantini D., Sivertsen B., Gehrman P., Lydersen S., Güzey I.C.

BMC Medical Genetics. 2019; 20:179-186.

The purpose of the study was to investigate whether SNPs previously reported to be associated with sleep-related phenotypes are associated also with different patterns of symptoms of insomnia. We used data from 3602 HUNT participants experiencing nocturnal symptoms of insomnia in all their possible combinations and 2427 participants who reported no symptoms. Controls were compared to each pattern of symptoms for frequencies of each of 58 SNPs, selected from previous studies on sleep-traits.

RESULTS:

- Association analyses for 16 SNPs had statistically significant results. Two SNPs were associated with two patterns of symptoms making the significant associations 18 in total.
- Seven SNPs presented also relevant odd ratio, however, after correction for multiple statistical testing none of the odd ratios were statistically significant.

Table 2: The 18 SNPs that were associated to different patterns of symptoms of insomnia and their previous associations.

SNP	Ref. allele	Other allele	Gene	Symptoms sub-group	B	OR	95% CI	p-value	Previous associations
rs10493596	T	C	AK5	SOI+TI	-0.82	0.45	[0.3 to 0.8]	0.004	Morning chronotype [135]
rs10823607	T	C	ADAMTS14	SOI+TI	-0.71	0.5	[0.2 to 0.9]	0.039	Sleep duration [126]
rs113851554	T	G	MEIS1	SOI+MI	0.4	1.5	[1.1 to 2]	0.007	Insomnia symptoms [128]
				SOI+MI+TI	0.36	1.4	[1.1 to 2]	0.007	
rs11706236	G	A	CACNA2D3	MI+TI	-0.33	0.7	[0.5 to 0.9]	0.009	Caffeine related insomnia [154]
rs12471454	T	C	SATB2	TI	-0.26	0.8	[0.6 to 1]	0.029	Insomnia [126]
rs12927162	G	A	TOX3	MI	-0.15	0.9	[0.7 to 1]	0.036	Morning chronotype [135]
rs1823125	G	A	PAX8	MI	0.16	1.2	[1 to 1.4]	0.033	Sleep duration [134]
rs1940013	T	C	OPCML	SOI	0.19	1.2	[1 to 1.5]	0.037	Usual bedtime 133
rs2221285	T	C	ESRRG	SOI+TI	-0.53	0.8	[0.7 to 1]	0.027	Sleep duration 134
rs2287838	G	A	PIN1	SOI	-0.22	0.8	[0.7 to 1]	0.022	Sleep duration 134
rs2302729	T	C	CACNA1C	SOI+TI	0.64	1.9	[1.2 to 3]	0.009	Sleep latency 126
rs34714364	T	G	APH1A	MI	0.16	1.18	[1 to 1.4]	0.041	Morning chronotype135
rs55694368	T	G	PER2	MI	-0.19	0.83	[0.7 to 1]	0.043	Morning chronotype135
rs6437122	G	C	UPP2	SOI+MI+TI	-0.34	0.7	[0.5 to 0.9]	0.015	Sleep duration 134
rs9517132	T	C	RANBP5	SOI	-0.22	0.8	[0.7 to 1]	0.018	Usual sleep duration 134
rs9804200	C	T	EBF3	MI+TI	-0.21	0.81	[0.7 to 1]	0.044	Usual bedtime126

SOI=sleep onset insomnia. MI=maintenance insomnia. TI=terminal insomnia.

4.3 Review of study 3

Differences in anxiety levels among symptoms of insomnia. The HUNT study.
Bragantini D., Sivertsen B., Gehrman P., Lydersen S., Güzey I.C.
Sleep Health. 2019; 5(4):370-375.

The aim of this study was to report and compare anxiety levels as measured with HADS-A among subjects reporting different patterns of symptoms of insomnia. For this purpose, we used 4317 cases, reporting at least one symptom of insomnia and 3616 controls, experiencing none of them. We compared mean HADS-A score among cases and controls and among individuals with different combinations of symptoms. Cases were divided in four standardized levels by increasing HADS score and the distribution of symptoms of insomnia among the levels was analysed.

RESULTS:

- The results of HADS-A differed significantly among the patterns of symptoms of insomnia after controlling for sex and age ($F(6, 4317)=43.92$, $p<0.001$);
- Participants reporting all three insomnia symptoms had the highest anxiety score (M=6.8, SD=4.3) followed in decreasing order by sleep onset insomnia with terminal insomnia (M=6.7, SD=4.0), sleep onset insomnia with sleep maintenance insomnia (M=6.3, SD=3.8), sleep onset insomnia only (M=5.8, SD=3.7), sleep maintenance insomnia with terminal insomnia (M=5.6, SD=3.4), terminal insomnia (M=5.2, SD=3.4) and sleep maintenance insomnia only (M=4.5, SD=3);
- Mean HADS-A score for maintenance insomnia alone was significantly lower ($p<0.05$) than the other patterns of symptoms except terminal insomnia alone.

4.4 Review of study 4

Epidemiological differences in levels of depressive signs among nocturnal symptoms of insomnia; results from the HUNT study.

Bragantini D., Sivertsen B., Gehrman P., Lydersen S., Güzey I.C.

Sleep science and practice. 2020;

The purpose of this study was to determine the level of depression as measure by the HADS-D in nocturnal symptoms of insomnia as defined in the DSM-5, considering all existing patterns. We compared HADS-D levels between 4317 HUNT3 participants who reported at least one symptom of insomnia and 3616 individuals who did not report any symptoms. Cases were further divided into seven subgroups according to the combination of symptoms they reported, and HADS-D results were compared among these subgroups. Differences in the distribution of patterns of symptoms among four standardized levels of increasing HADS-D score were also evaluated.

RESULTS:

- HADS-D scores differed significantly among the patterns of symptoms ($F(6, 4317)=27.35$, $p<0.001$);
- Participants reporting all three insomnia symptoms had the highest depression score ($M=5.2$, $SD=3.6$), followed in decreasing order by sleep onset problems with terminal insomnia ($M=5$, $SD=3.4$), sleep onset insomnia with sleep maintenance insomnia ($M=4.6$, $SD=3.2$), sleep maintenance insomnia with terminal insomnia ($M=4.3$, $SD=3.1$), terminal insomnia ($M=4.1$, $SD=3$), sleep onset insomnia only ($M=4$, $SD=3.2$), and sleep maintenance insomnia only ($M=3.4$, $SD=2.9$);
- Individuals reporting only maintenance insomnia scored lower than those experiencing any of the other patterns of symptoms;
- Inclusion of sex and age as covariates $F(6, 4317) =28.7$, $p<0.001$) did not result in statistically significant changes in the mean differences among the groups.

- In the “Normal” HADS-D group 33% experienced maintenance insomnia. This percentage was higher than for all the other groups ($p<0.001$). In the “Severe” group 41% of the individuals reported all three symptoms of insomnia.

5. General discussion

The aim of this thesis was to investigate differences in genetic and psychological factors among individuals presenting different nocturnal symptoms of insomnia. Using a sample form the HUNT3 cohort, we analysed differences among the combinations of symptoms 1) in their association with SNPs in circadian genes; 2) in their association with SNPs previously reported as linked with sleep phenotypes; 3) in levels of anxiety and 4) symptoms of depression.

5.1 Symptoms of insomnia as self-standing entities

We identified differences in genetic and psychological aspects of different patterns of symptoms of insomnia, although some of those were statistically non-significant after correction for multiple testing. The validity of such correction in the context of biological research will be discussed later in this chapter.

No previous genetic studies have investigated all possible patterns of nocturnal symptoms of insomnia separately and therefore we cannot compare our findings directly with other studies. When it comes to differences in psychopathologic characteristics among the symptoms of insomnia, only few studies have reported this aspect. I could identify three studies which analysed differences in anxiety and depression among onset, maintenance and combined insomnia[59,62,148] . The differences in the psychopathological aspects varied among the studies and variations in the definition of the symptoms and tools used makes it difficult to directly compare these findings with ours.

A neuroimaging study reported thinning of the OFC only in patients experiencing early morning awakenings, suggesting that specific areas of the brain may superintend specific symptoms[146]. ORF thinning also characterized patients with depression. These pieces of evidence together could help support the clinical notion that patients with the melancholic subtype of MDD experience early morning awakenings[2].

The limited number of studies investigating differences among the symptoms of insomnia is the product of a "diagnostic" approach to research in this field. As for other psychiatric diseases, research on insomnia is focused on the use of clinical diagnoses. These are extremely useful in clinical settings but can be limiting in research.

In genetics for example, to have a well-defined phenotype is one of the major limitation of the field[156,157]. For this reason, *deep phenotyping*, meaning finding biologically meaningful subtypes of a diagnosis, have been stressed by many authors. Geneticist Gary Churchill efficiently expressed the importance of deep-phenotyping referring to diabetes: "There are a hundred ways to be diabetic, involving different processes in the pancreas, liver, muscle, brain and fat. Genetic studies lose statistical power by looking at a conglomeration of underlying causes"[156].

For many somatic diseases, such as diabetes and cancer, deep phenotyping is eased by the presence of a vast amount of biochemical and molecular information. These information are used to characterize different sub-phenotypes or even to identify them, in a bottom-up way (i.e. molecular sub-typing)[158]. Diagnosis and treatments options are improved enormously for these diseases, where the quest for "personalized medicine" is constantly advancing (225 articles in 2009, 2250 only in 2019). Conversely, for psychiatric conditions we have mostly descriptive phenotypes especially due to difficulties in accessing the brain[156]. The physiological complexity of this organ have made the unravelling of its molecular pathology even more challenging[159].

One could also object that the course of chronic insomnia often presents changes over time, with some symptoms subduing and others emerging in the same individual[59,160]. This was suggested as evidence for confuting the hypothesis that the symptoms indicate different entities. However, these changes in symptoms over time do not exclude the presence of specific genetic profiles, distinctive consequences and implications for each symptom. Hypothetically, the symptoms that an individual is more likely to experience could be limited by his genotype

while dynamic environmental cues decide which symptoms will emerge at different given times.

5.1.1 Differences in genetic predisposition

Results from studies 1 and 2 were overall neutral (e.g. statistically non-significant). After correction for multiple testing, none of the SNPs in circadian genes nor those previously associated with sleep phenotypes remained statistically significant.

5.1.1.1 Circadian genes and symptoms of insomnia

In study 1, before correcting for multiple hypothesis testing, 26 SNPs were significantly associated with patterns of symptoms of insomnia. Nine variations were predicted to lay on a functional site.

Rs3824872 and rs7121775 on *CRY2*, rs2518023, rs2585408, rs3027160 on *PER1*; rs4663866 on *PER2* and rs4795424 on *REV-ERBa* are predicted[XV] to be on binding sites[XVI] for transcription factors[XVII]. SNPs in these regulatory sites can in some cases increase or decrease the efficiency of the interaction between proteins and DNA. This may have an effect on the amount of the corresponding proteins that are produced. Speculatively, slower production of circadian products and consequent delay in reaching the concentrations necessary to activate the feedback loop could account for sleep disruption and other conditions.

Rs3027178 on *PER1* were predicted to be on a splicing site. These are portion of a gene that are recognized by a complex of enzymes, the *spliceosome*. This complex has the function to split introns (i.e. non-coding regions of a gene) from the molecules of mRNA. Variations in these areas can interfere with the action of the

[XV] All prediction on SNPs activity are made by the bioinformatics tool SNPinfo Web Server[167].
[XVI] **Binding site**: portion of a gene that are recognized and docked by enzymes that aid functions such as DNA replication and transcription.
[XVII] **Transcription factors**: enzymes that bind the sequence of DNA in binding sites called promoters and enhancers and aid the transcription of DNA to mRNA.

spliceosome leading to spurious mRNA that is eventually destroyed or translated into faulty proteins[161].

One variation on *PER2*, rs934945, is predicted to be a missense mutation. These variations reveal their gravity at the protein level by changing the sequence of amino acids. The consequences of missense mutations can vary from extremely severe to non-detectable, depending on the position in the sequence and the chemical properties of the new amino acid. In our case, the presence of the variation in 21% of the population suggests that the effect of this variation must be mild (i.e. missense variants with severe effects are much rarer).

Overall, we noted that some symptoms clustered around different circadian genes. Reporting all symptoms of insomnia together was exclusively associated with gene *PER3*, early morning awakenings with genes *CRY1* and *CRY2*, while sleep onset problems with gene *ARNTL*. Transcription of these genes is at its maximum in different parts of the day and therefore it is possible that they concur in regulating different aspects of sleep[25]. Abnormal functioning of the genes might in turn produce different symptoms of insomnia.

Some previous studies had found polymorphic loci in circadian genes that increased the risk to experience insomnia. However, these studies were conducted almost exclusively in psychiatric samples, which may constitute a source of bias. Psychiatric conditions could work as mediators in the relationship between genetic profile and insomnia. Indeed, psychiatric conditions are also studied in the prospective of being "circadian dysfunctions" in which the timing of action of neurotransmitters is dysregulated. The periodically changing symptoms of depression, that exacerbates in the morning, and bipolar disorder, characterized by periodic cycling of symptoms are thought as the result of faulty circadian rhythmicity[54].

PER3: the peripheral regulator

PER3 is transcribed under the promoting effect of ARNTL-CLOCK. The resulting protein has among its roles to repress the transcription of the gene *PER3*.

Transcription of PER3 peaks among 4 to 8 hours after sunrise and then it starts blocking its production. The relationship between presenting all three symptoms of insomnia and PER3 speculatively may lay in a desynchronization of the peripheral oscillators of the brain from the master clock.

Previous studies reported involvement of *PER3* in sleep phenotypes such as sleep homeostasis, cognitive functions after sleep loss[33], chronotype[135,162,163] and psychiatric conditions such as bipolar disorder[119,164] and depression[164]. Moreover, animal studies suggests that PER3 has more important role in the peripheral areas of the brain while its role may be limited in the SNC[165]. Silencing PER3 in mice produces slight effects on the timekeeping activity of the master clock. On the other hand, peripheral oscillators present an advance in phase compared to wild type (e.g. "normal") mice[166]. All the areas of the human brain are usually synchronized with the master clock[25] and desynchronization seems to concur in the developing of neuropsychiatric diseases. Desynchronization of peripheral areas has already been reported as occurring in the brains of individuals affected by psychiatric and neurological conditions such as MDD[167], Alzheimer's and Parkinson's[168].

ARNTL and rhythm delay

Polymorphisms in gene *ARNTL* were mostly associated with sleep onset insomnia suggesting that a delay in the rhythm may be involved. The protein coded by gene *ARNTL* couples with protein CLOCK to work as transcription factor for several genes, including its own repressors PER and CRY. ARNTL transcription peaks at night and is involved in transcription of genes involved in wakefulness[23]. The effect of SNPs on this gene may also produce proteins unable to pursue specific regulatory duties that will interfere with sleep. Experimental evidence showed how silencing *ARNTL* from neurons in the tuberomamillary nucleus of mice brains did not disrupt circadian rhythmicity but produced constantly elevated histamine levels. Histamine promotes wakefulness and indeed the animals demonstrated fragmented sleep and altered sleep architecture[169]. KO mice for *ARNTL* homologous gene *BMAL1* showed among other phenotypes shorter bouts of sleep

and shorter total sleep length[117] This circadian gene was also previously reported in several studies on chronotype[135] and cycling mood disorders[170]. One SNP in particular, rs3816358, was associated with later sleep onset time in a sample of elderly people[171] and was among our statistically significant results.

CRY genes and early morning awakening

In our study, seven SNPs on genes *CRY 1* and *2* were associated with early morning awakenings. Of notice, two of these variations, rs3824872 and rs7121775 on *CRY2* are predicted to be on binding site for transcription factors. *CRY* gene products works as suppressor together with PER proteins for the ARNTL-CLOCK dimer. Recent KO experimental reports on human cells showed how KO cells for *CRY1* had shorter periods with lower amplitude as measured by *ARNTL* expression. The periodicity is lost after few days and the KO cells become arrhythmic. Conversely, cells with silenced *CRY2* had longer periods with only slightly reduced amplitude. Finally, cells lacking both are completely arrhythmic. These studies supports previous animal studies on how these two genes cooperate to maintain circadian periodicity[172]. A mutation on the *CRY1* is involved in Familial Delayed Sleep Disorder, a condition characterized by a phase advance in one's circadian rhythm. People presenting with the condition will often fall asleep only at late hours and sleep sounder throughout the day than healthy individuals[173]. The mutation makes the CRY1 protein a more effective transcription inhibitor interfering with the transcription action of ARNTL-CLOCK and therefore delaying the whole machinery. Conversely, in our study the association of *CRY 1* and *2* with early morning awakenings, could indicate phase advance rather than delay. It is possible that the mutation we investigated weakened the effect of CRY1 creating a shorter circadian period and therefore earlier awakenings.

5.1.1.2 Pleiotropy of symptoms of insomnia and sleep phenotypes

Regarding results from study 2, 16 SNPs were significant in 17 association analyses (one SNP was associated to two patterns). Only three SNPs were predicted to be functional: rs10823607 on *ADAMTS14* (on an *enhancing splicing*

site), rs12927162 on *TOX3* (on Transcription Factor Binding Site) and rs34714364 on *APH1A* (on an enhancing splicing site). For what concern the other SNPs, some belong to genes with plausible biological backgrounds while the role of others remains uncertain. Among the latter we find indeed *ADAMTS14* involved in the pathway of collagen formation[174] and *APH1A* interacting with enzymes involved in the metabolism of the peptide Amyloid beta, crucial in the pathogenesis of Alzheimer's disease[175].

SNP rs10493596 close to the *AK5* (Adenylate Kinase 5) gene was one of the variations with lower p-value (before FDR correction) and promising OR. Adenylate kinases are involved in catalysing the transfer of phosphate groups among adenine nucleotides to produce Adenosine triphosphate (ATP). Interestingly *AK5* is expressed exclusively in the brain, which makes its involvement in neurological phenotypes such as sleep less random. In our study, rs10493596 was associated with difficulties falling asleep in combination with early morning awakenings while previously it was reported as more frequent in morning chronotypes[135].

Difficulties in falling asleep with early morning awakenings showed also an association with gene *CACNA1C* (Calcium Voltage-Gated Channel Subunit Alpha1 C). This gene code for a subunit of a calcium channel involved in cellular signalling induced by membrane depolarization; in the brain, they are located in the dendritic spines where they transmit incoming signals. Specifically, the variation that we reported rs2302729, was previously associated with sleep quality and latency[134]. Absence of this receptor subunit creates severe phenotypes in mice resembling neuropsychiatric disorders. Both positive symptoms (hyperactivity and anxiety) and negative ones (decreased sociability and cognitive abilities) were described in KO mice for *CACNA1C*[176]. In humans, rare mutations in this gene were found [177].

Among our highest hits the presence of a polymorphism on gene *MEIS1* is noteworthy. *MEIS1* is involved in restless leg syndrome (RLS) [178-180] but also reported to be associated with insomnia symptoms[127,128,178]. *MEIS1* expression is

decreased in the brain of RLS patients and there is evidence for its involvement in iron metabolism in the brain (i.e. low iron levels in the brain is one of the most accredited causes of RLS)[181]. In our study, combinations of sleep onset problems with maintenance insomnia and all three symptoms of insomnia together showed low *p*-values (0.01 and 0.02 respectively) and discrete odds ratio (1.5 and 1.4) for the T allele of rs113851554, in agreement with previous studies.

A polymorphism in gene *TOX3* (TOX High Mobility Group Box Family Member 3), rs12927162, resulted in significant association with maintenance insomnia. Previous reports of this SNP come from studies on chronotype and accelerometer measures. The A allele increased the chances of being a morning person[135] but at the same time of presenting a circadian phase delay[182]. Moreover, as *MEIS1*, *TOX3* was also associated with RLS[179,183], which strengthen the notion that the insomnia phenotypes may be "contaminated". However, the proteins coded by these genes seem to have generic functions and could therefore influence different traits. TOX3 is a calcium-driven transcription factor largely expressed in neurons[184] but studies on its role in sleep physiology have not been conducted yet.

The fact that genes as *MEIS1* and *TOX3*, associated with RLS and insomnia could be a case of pleiotropy. The products of these genes could serve a very general function or several ones and therefore give a signal in genetic studies of both RLS and sleep phenotypes. Alternatively, the hypothesis that insomnia and RLS may be overlapping phenotypes not easy to discern have also been proposed[128].

Another gene with no reported role in sleep physiology but replicated in few genetic studies on sleep phenotypes is *PAX8*. In particular, rs1823125 was associated with sleep duration[134,185] and efficiency[182] in two different samples, while in our study gave a signal for maintenance insomnia. Even if the biological role of *PAX8* appear to be related to the embryonal development of thyroid and kidneys, it is possible that it serves other functions not yet reported. However, rs1823125 is located in an intragenic region and is said to "belong to" *PAX8*, being this the closest gene. Speculatively, the variation could belong to the regulatory region for another gene or several others[186]. In a third possible scenario, as for

MEIS1 and *TOX3*, other diseases could mediate the relationship between *PAX8* and sleep phenotypes.

5.1.1.3 Multiple testing in biology

The presence of a pattern of genes and symptoms in study 2 and of some biological and replication evidence in study 3 could be a sign that we were overly conservative when correcting for multiple testing. In the words of Rothman: *"[…] when scientists are studying biological relations rather than random numbers, the premise that type I errors are the major concern may be wrong"*. Rothman invites to use a moderate caution in studies such ours. One may want to be more severe when testing for example the trustworthiness of psychic powers and less stringent when observing physiological effects induced by a designed new drug. The genes we selected in both study 1 and 2 are implicated in sleep and therefore their role in insomnia would not be surprising. As casual as it sounds to discuss the validity of methods and findings in these terms, it is the theory behind Bayesian statistics. Our prior knowledge together with information we get from the test statistics should weights the likelihood of our hypotheses[187].

5.1.2 Differences in psychopathological traits

Study 3 and 4 focused on psychological elements that are common in insomnia: anxiety and symptoms of depression. The distress of the objective or subjective loss of sleep may lead to develop these comorbid conditions. At the same time, presenting psychological distress could interfere with sleep. Overall, our results show that anxiety levels differ among persons with different pattern of symptoms of insomnia while depressive symptoms do not differ as much.

The differences between the results of study 3 and 4 cannot be explained in terms of different qualities of the two scales. Both HADS scales are reported to perform moderately well regarding both sensitivity and specificity (when the cut-off value is set to 8) and they correlate well with responses from other questionnaires on psychological distress (Beck DI, State Trait Anxiety Inventory etc.)[188].

In both studies, HADS scores were significantly higher for cases than controls, in line with the current knowledge. Large meta-analytic studies have confirmed that anxiety[137] and depression[136] increases the risk of comorbid sleep difficulties. The results were in both cases over the minimal important difference (MID) estimated specifically for each HADS scale. For HADS-A the difference was 3.1 (MID=1.5-2.5) [189,190] while for HADS-D it was 2.2 (MID=1.9 -2.3)[189]. These results give a mild support to the validity of our selection method and to the items included in the HUNT questionnaire to differentiate people with insomnia-like disturbance from good sleepers.

Another finding that match in the two studies is that maintenance insomnia alone has the lower scores among the patterns of symptoms. This is possibly in agreement with experimental studies on induced sleep fragmentation. Results from these studies show that cortisol levels rise abruptly the first time the subject is awaken but the response decrease rapidly throughout the night and is normalized the morning after the experiment[191,192]. The reason of this may lay in the phase of sleep that is interrupted (REM versus NREM) but also in the fact that although fragmented some amount of sleep is obtained. Even if inconvenient, sleep fragmentation is in fact less detrimental than partial sleep deprivation on physiological parameters and cognition[193]. Moreover, some studies have confuted the notion that people with insomnia have more awakenings than normal, showing instead that they have longer awakenings. This suggests that maintenance insomnia is indeed only a "temporal variant" of sleep onset insomnia[194]. In other words, the problems is not the awakenings but the difficulty in falling asleep once awaken.

Another possibility is that, in some cases, maintenance insomnia is in fact the manifestation of sympathetic arousal given by the struggle to breath (i.e. given by sleep apnoea), of disturbance given by periodic limb movements or by other conditions. At the same time, in many studies this symptoms of insomnia is reported as the most frequent[59,148] and therefore it is unlikely that the majority of respondents suffer from SA, RLS and medical other conditions.

In both studies, we found a difference in mean scores among women and men. While in study 3, anxiety was slightly higher for women without distinction among the symptoms of insomnia, in study 4 depression was more elevated for men in the same way. As we did not include any variable that could further explain sex-related differences and as the differences do not have a clinical relevance, these results are only descriptive.

The inverted trend that sees males scoring higher than women in HADS-D is a known phenomenon. This trend is in contrast with what is found in the literature about level of depressive symptoms and incidence of clinical depression among the sexes. Women are generally more predisposed to develop depression (OR~=2)[77] and display depressive symptoms (OR=1.6)[77], in particular somatic ones (e.g.: tension pain, CVD and gastrointestinal issues[195]). HADS-D does not cover this class of symptoms and it may therefore underestimate the level of depression in women[196].

5.1.2.1 Anxiety

Anxiety levels were significantly higher for people experiencing difficulties falling asleep, either alone or in combination with other symptoms. Individuals reporting only maintenance insomnia showed the lowest mean levels of anxiety among the cases, while reporting all symptoms together was associated with the highest.

Although statistically significant, the differences among single symptoms cannot be considered clinically relevant when considering the MID. The mean anxiety level for respondents reporting only sleep onset insomnia were 1.3 points higher than those reporting maintenance insomnia, while reporting terminal insomnia showed only 0.7-point mean difference. These values are not clinically significant but from an observational perspective they invite further investigation. Unfortunately, lack of information about several other sleep parameters leaves only room for speculation about the reasons of these differences.

Objective short sleep duration is said to increase insomnia severity, as opposed to paradoxical insomnia[197]. It is possible that the awakenings are very short and

therefore are not interfering with sleep length and the maintenance insomnia group is not homogenous in this sense. Including information such as WASO and the number of interruptions per night could reveal the presence of subgroups with different characteristics. A second possibility is that in some cases, reported fragmented sleep is in fact paradoxical insomnia where sleep is perceived as wakefulness[84]. Reported fragmented sleep could not interfere really with sleep, allowing all the physiological processes to be completed. A study from 2019 brought evidence of a correlation between reduced SWS and higher levels of anxiety[198]. This could also suggest that fragmentation in the majority of cases do not affect SWS but happens during REM or other NREM stages.

Paradoxical insomnia, however, can also apply to sleep onset insomnia. Comparing EEG recordings and reported SOI showed that fragmentation at the beginning of sleep is often perceived as not having slept at all and [90]. However, inability to fall asleep due to anxiety can be increased by the fear of not getting enough sleep[91]. Over time, this shapes a distorted association between lying in bed and struggling to sleep that interfere with falling asleep[199]. In any case, we do not have elements to determine the basis of the differences among these symptoms.

The literature gives scarce support in the analysis of our results as only three studies analysed differences in anxiety measures among sleep onset and maintenance insomnia[59,62,148]. In contrast to ours, two early studies presented rather small sample sizes, one of these and the third one used different tools from ours to measure anxiety. Persons with sleep onset insomnia did not display different mean HADS scores (N=30)[62], State Trait Anxiety Inventory (STAI, N=149)[148] or Beck Anxiety Inventory (BAI, N=954)[59] than those with maintenance insomnia.

The level of anxiety experienced in the presence of one symptom of insomnia did not demonstrate to follow an "additive law". Anxiety levels associated with sleep onset insomnia were not affected by the presence of a second symptom of insomnia. Persons who had maintenance and early morning awakenings problems in addition had also outcomes similar to those with sleep onset insomnia only. These

results are also different from what presented in a study in which individuals reporting both onset and maintenance problems had higher levels of anxiety that those experiencing only one symptom[59].

In our sample, a considerable number (N=642) responded that they experienced all three symptoms several nights per week. This group scored the absolute highest in the HADS-A, but the mean value was not statistically different from the group reporting sleep onset insomnia alone or in combination with other symptoms. This seems to indicate that sleep onset problems contribute most to anxiety.

This finding could help improve the existent therapeutic interventions for patients with sleep onset problems. Psychotherapy in the form of Cognitive Behavioural therapy for insomnia (CBT-I) is only relatively efficacious in relieving anxiety. Similarly focusing exclusively on anxiety did not resolve insomnia adequately[200]. For now, there are no studies examining differences among the symptoms in effectiveness of therapeutic interventions. One study evaluating the use of relaxing techniques to ease bodily tension found an effect on sleep onset latency[143]. The authors were expecting to evaluate the intervention also on maintenance problems. However, polysomnography did not identify any case of maintenance insomnia. Even if the authors designed the study in order to include only participants with "psychological insomnia", they did not include any subjective reports of symptoms in the analysis.

5.1.2.2 Depressive symptoms

For depressive symptoms, the situation was different as the differences we found among the symptoms of insomnia were far smaller than the MID. Among the single symptoms of insomnia, maintenance insomnia was the least affected by depressive symptoms (M=3.4), while showing symptoms at the extremes of the night had the highest (SOI=4.0, TI=4.1). These did not differ from each other but experiencing them at the same time was associated with a higher mean HADS score (M=5).

These two symptoms could be present concomitantly as result of a common pathological background or emerge independently from rarely co-occurring factors. Hypothetically, these two symptoms of insomnia and depression could be connected in a rapidly sequential way. Terminal insomnia could emerge concomitantly to depression. Overlapping grey matter reduction in the OFC between early morning awakenings and depression indicates that, at least in some cases, these conditions may have the same neuropathological profile[63,146]. Worsening of depression may trigger sleep onset problems[61]. The low prevalence of this pattern of symptoms could also indicate an unidentified rare condition with symptoms such as reduced ability to sleep and depression, but this notion is for now only speculative.

Interestingly, to report all three symptoms of insomnia at the same time did not rise the HADS-D score any further (M=5.2). Previous studies have also reported that differences in measures of depressive symptoms among single symptoms of insomnia were not statistically significant[61,148].

Contrariwise, in agreement with our study, "combined insomnia" (i.e. sleep onset and maintenance/terminal insomnia) was associated with higher levels of depressive symptoms[59,148]. As mentioned earlier, maintenance insomnia may be provoked by of somatic conditions and therefore these patterns of symptoms of insomnia could be in "real" sleep onset insomnia concomitant with another condition. The psychological burden of the two disorders simultaneously may interact fuelling depressive symptoms.

Insomnia is common comorbidity of depression[201] but the relationship seems to have some symptoms-specific aspects. Indeed, "early morning awakenings" is listed in the DSM-5 among the sign of *melancholic depression,* a sub-type of MDD[XVIII]. Melancholic depression is characterized by anhedonia and lack of mood reactivity. Although HADS is not a diagnostic tool, HADS-D is considered well designed to measure specifically the anhedonic aspects of depression as it includes

[XVIII] This notion derives seemingly from clinical empirical knowledge since the scientific articles on the topic that I could find refer always only to the DSM-5 and the manual itself does not cite any source.

items such as “I still enjoy the things I used to enjoy” and “I can enjoy a good book or radio or TV program”[202]. However, in our sample reporting early morning awakenings did not give distinguishable results. The percentage of respondents reporting this symptom was the same across the four groups of increasing HADS severity.

Individuals reporting symptoms of insomnia did have higher HADS-D score than the “good sleepers”. However, the difference between the two groups fell between the lower and upper limit of the estimate MID. A previous study on the HUNT3 sample found the OR of developing depression in participants who experienced symptoms of insomnia to be higher than other studies[203]. The difference is attributed to the fact that the study included daytime impairment to operationalize insomnia while other studies did not. It is possible that the decision to omit the item on sleepiness would have “diluted” the sample, including individuals who were not distressed by the symptoms.

5.1.3 Implications and future directions for research and practice

This thesis overall objective was to put on a critical light the assumption that the night-time symptoms of insomnia are equivalent in their characteristics, that they have the same weight when setting a diagnosis and that they will respond equally when designing a treatment. The findings presented in this thesis are just a glimpse to possible differences among the symptoms. Implications for the study of differences among the symptoms of insomnia are for now only speculations. However, continuing with this approach could benefit several aspects of both research and clinical practice.

5.1.3.1 Implications for sleep and insomnia research

Focusing on aspects that are characteristic of each symptom could not only reveal the factors impeding onset and maintenance of sleep, but also consequently help clarify several aspects of sleep physiology and neuropsychology. Examples from other conditions demonstrate that sub-phenotyping can give new inputs to research.

For example, neuroimaging exposed specific differences in the density of grey matter (GM) among patients with positive, negative and disorganized symptoms of schizophrenia[204]. Stratifying the results by symptoms allowed to show, for example, that positive symptoms were associated with reduced GM in the PFC, similarly to other conditions characterized by hallucinations (e.g. Bipolar disorder type I). This show that diseases that are considered separate in diagnostic manuals are in fact overlapping at a neuroanatomical level. Neuroimaging showed also differences among the symptoms of insomnia and neuroanatomical features specific for terminal insomnia comorbid with depression[63,146]. These differences may be the product of different genetic backgrounds that influence anatomical development and functioning of the brain, also in cooperation with different external stimuli.

Clarifying the characteristics for each symptom of insomnia will help both deepening the understanding of the neurobiological basis of sleep and how to improve treatment of insomnia by providing focused, personalized options.

5.1.3.2 Implications for the choice and design of therapeutic interventions

Identifying sub-phenotypes could bring advantages also to the design and implementation of therapeutic interventions. In example, speculatively, the neuroanatomical markers specific for the three subtypes of schizophrenia could allow early diagnosis and interventions that would benefit especially patients with negative symptoms, where loss of cognition can prove irreversible[205]. Similarly, identifying biological or psychopathological markers of insomnia could help prevent the worsening of the condition or the development of comorbidities.

The differences among the symptoms may reveal the possibility for tailored interventions for each pattern of symptoms. Existing therapeutic options for insomnia could increase their positive outcome rate and their long-term effect by targeting also specific associated psychological issues and personality traits.

For example, some of the findings presented in this thesis suggests that offering a therapeutic intervention for insomnia together with one designed to address

anxiety or depression could bring more benefits for individuals experiencing sleep onset insomnia. A hybrid intervention could either prevent the worsening of the condition and development mental distress or could boost improvement by covering the comorbid state.

It is possible that such treatment could help also maintenance insomnia, but it is possible that usual treatments would be enough to ameliorate the condition. Alternatively, identifying factors that are most relevant in maintenance insomnia may reveal that, for example, shorter treatment options are necessary for people experiencing this symptom of insomnia. This could be economically beneficial for both clients of private practitioners and the public health system.

5.1.3.3 Future studies: refined data collection and clinical samples

The knowledge reported in the present work is far from clarifying biological processes of sleep or being of use in a clinical setting but hopefully will inspire future research. In particular, dedicated studies designed to collect sleep related data with higher accuracy could bring progress to the field of sleep biology and medicine. Likewise, applying this approach to clinical samples in which the symptoms of insomnia and comorbid psychopathologies are more severe, could help different characteristics and implications of each symptoms to emerge. A clinical setting could allow also to collect information through more reliable tools such as structured and semi-structured interviews conducted by professionals.

5.2 Methodological issues

5.2.1 The HUNT3 sample

The studies included in this thesis were conducted using data from the HUNT3 study. Using data from a general population, that includes several hundred variables (millions if we include the genetic data) and several thousand participants can, however, still present with some shortcomings.

Of the over 93,000 people invited to participate in HUNT3, 54% attended the study[206]. With information on half of the population lacking, it could be easy to conclude that the participation rate is only moderate. However, participation

rate have been questioned as a measure to determine the quality and validity of a sample as studies with low participation rates (as low as below 20%) demonstrated to give the same estimates as studies with higher rates[207]. Besides, looking beyond the participation rate one will realize that the HUNT3 sample includes still over 50,000 individuals.

Investigations on other health related cohorts suggested also that the individuals with high income and high level of education are more represented than in the general population in these studies[208]. This is true also for the HUNT3 study in which the non-participants presented also higher mortality and prevalence of chronic diseases[209].

Finally, the population of the region of North-Trøndelag was chosen for its relative isolation (e.g. very low immigration and emigration rates), an element that allows to reduce the influence of unknown external factors (genetics backgrounds, cultural factors, etc.)[206]. Especially for genetic studies, isolates give the advantage of a homogenous genetic profile where the frequency of some disease-related SNPs could be *enriched* (e.g. occurring more frequently than other populations). This phenomenon is a consequence of a certain level of inbreeding of the individuals in the population that reduces genetic variability (e.g. relatives have similar genetic profiles). This is both an advantage, for it allows to find genetic associations that are elusive in population with more genetic complexity, but also a disadvantage because the result may not be generalizable to other populations. However, this is a problem of all genetic epidemiological studies that is only more accentuated in population isolates[210].

5.2.2 Definition of the phenotype "symptoms of insomnia"

The HUNT questionnaire about sleep as a whole is reported to be a good tool to identify people with sleep disturbances[211]. However, the single items of the questionnaire had only moderate reliability when compared to an interview one to two months later. Anyway, long time gaps between test and interview could explain the low reliability.

One of the major limitations of this study is the operationalization of the symptoms of insomnia with the data available in the HUNT study. The goal of the HUNT to collect information on several health-related matters from thousands of individuals, imposed restrictions on the number of items in the questionnaires. For this reason, the definition of insomnia we used in this study was based on two criteria listed in the DSM-V: the presence of at least one nocturnal symptom and the criteria of frequency of the disturbance (three times a week in last three months). The phrasing of the questions did not specify other criteria for insomnia such as the lack of a clear somatic problem (i.e. pain) or external reason (i.e. being a caretaker) for disrupted sleep. Moreover, the questionnaire did not include any item investigating if the symptoms bothered the respondent nor inquire about the quality or quantity of sleep.

In order to reduce bias in defining the phenotype, we used questions inquiring about the presence of symptoms of sleep apnoea and snoring to select the participants. However, we considered other questions about sleep related matters not sufficiently well formulated to have discriminant power.

One question investigating the presence of sleepiness during daytime was not taken into account to select cases. Clinically relevant daytime impairment is listed among the criteria for the diagnosis of insomnia but usually it manifest itself in form of tiredness more than sleepiness[212,213]. In fact excessive daytime sleepiness (ESD) is a more common symptom of sleep apnoea[212] rather than insomnia. Moreover, the question proposed in HUNT is only in low agreement (k=0.27) with a more extensive tool such as the Epworth Sleepiness Scale (ESS) [211,214].

Another question that we excluded from the including criteria for the study asked about "an uncomfortable or pins and needles feeling" in the legs. This item was clearly designed to investigate RLS but we argue that it was too vague to be used as a proxy for the disorder. In RLS, the feeling is often described as urge to move the legs due to a crawling sensation in the muscle. The symptoms often exacerbate at night or during rest therefore affecting sleep. Unfortunately, the item used in

the HUNT study was phrased in a way that is open for many different conditions affecting the legs.

5.2.3 Genetic analyses

The analyses we conducted were single SNP analyses. However, insomnia is a complex disease, which is most likely not a product of a single genetic variation. For this reason, the methodology we chose could be ineffective when several SNPs interact in producing a phenotype. However, analysing the effects of the interaction of many variations (epistatic interaction) is computationally cumbersome and often relays on heuristic methods (i.e. trial-and-error methods based on practical common sense) to identify the number of interacting SNPs[215].

The analysis of SNPs previously associated with sleep phenotypes could have been more reliable using the method called "genome-wide genetic correlation"[216]. This approach consists in the identification of overlapping genetic areas by comparing GWAS results for two traits[216]. Unfortunately, the HUNT databank does not include variables for sleep related traits such as sleep timing, normal sleep duration or chronotype. Moreover, a GWAS study requires considerably more funding than the "select and test" method we used.

5.2.4 Measures of anxiety and depression

Anxiety and symptoms of depression were measured using the HADS questionnaire[155]. The questionnaire was designed to evaluate these psychological issues among hospitalized patients, but it has good reliability and internal consistency also in samples from general populations[188,217-219]. When it comes to specificity, however, the situation is slightly different for the two subscales[188]. Although some studies reported excellent specificity to recognize both MDD and GAD[219], others found better results for HADS-A than HADS-D[220]. The latter neglects indeed somatic symptoms, presence of sleep disturbances and altered appetite that are common manifestations of depression[221]. In 2012, some researcher even suggested to discontinue the use of HADS on basis of labile semantic meaning of some of the items and lack of specificity of the scales[221].

The notion that HADS lacks specify was introduced by a study that reported that its items loaded onto two latent factors, *autonomic anxiety* and *anhedonic depression*. Correlation among the items in the two scales was assumed to load onto a third, high-order factor, *negative affectivity (NA)* [202]. These findings were confuted by a meta-analysis that supported instead a *Bifactor model*, characterized by a broad general factor, *general distress* (G), and narrower conceptual factors, anxiety and depression. The difference between the high-order and the Bifactor models is that in the latter the third factor is on the same level as the other two and variance can be calculated independently for each factor[222]. This allows for the scores of the two scales to be used as variables in statistical models.

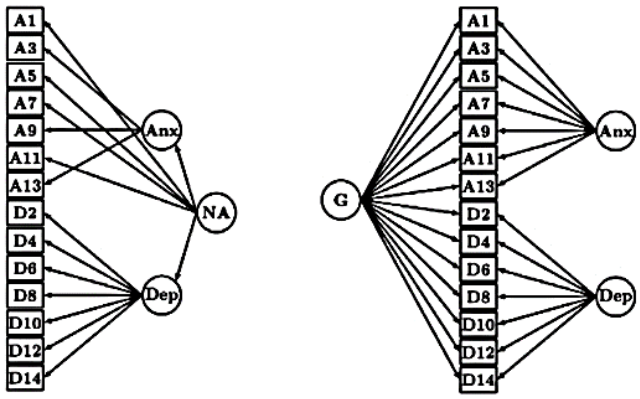

Figure 4. The *high-order model* (on the left) and the *Bifactor model* (on the right). NA=negative affectivity. G=general distress. Anx=anxiety. Dep=depression. The alphanumeric codes in the boxes indicated the questionnaire items number and the scale they belong to (A=anxiety, D=depression).

Nevertheless, the general distress factor expresses the presence of correlation between the scales [223]. For this reason, some authors invite to the use of the subscales of HADS only in research. Conversely, in clinical settings, they advise to limit the use only to total HADS, to evaluate the presence general psychological distress[223]. However, these considerations are based on meta-analytic results that included studies with different criteria. In several studies the cut-off value used to differentiate clinical depression from health is as low as four. However, a better equilibrium between sensitivity and specificity is reached by setting eight as a critical value[155,188].

Nonetheless, HADS is still widely used and its popularity is never declining as a search of a literature database such PubMed will demonstrate. As a clinical tool it

could be insufficient but it can be considered a fair compromise in a study such as ours, which covered a very large population, with epidemiological purposes.

6. Conclusions

The studies presented in this thesis provide a characterization of nocturnal symptoms of insomnia as different entities:

In the first study different patterns of nocturnal symptoms of insomnia were not associated with SNPs in circadian genes. However, before correction for multiple testing gene *PER3*, *CRY1* and *2* and *ARNTL* were almost exclusively associated with all symptoms together, terminal insomnia and sleep onset insomnia respectively.

In the second, the patterns of symptoms were not associated with SNPs previously reported in association with sleep traits.

In the third study, sleep onset insomnia was associated with higher levels of anxiety, both alone and in combination with other nocturnal symptoms.

In the fourth and last study, depression levels were higher in people with sleep onset insomnia combined with terminal insomnia. Those who also reported maintenance insomnia did not have a statistically significant higher mean in depression levels. This was in agreement with people reporting only maintenance insomnia presenting the lowest mean HADS-D score.

To conclude, this series of studies showed that the symptoms of insomnia differ in anxiety levels, with sleep onset insomnia contributing substantially in rising the distress. Evidence for differences in other factors are less convincing but still suggestive that the symptoms may have individual characteristics. Future studies focused on the individual symptoms of insomnia may help give a better understanding of this matter.

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Appendix

Paper 1

Paper 2

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RESEARCH ARTICLE Open Access

Genetic polymorphisms associated with sleep-related phenotypes; relationships with individual nocturnal symptoms of insomnia in the HUNT study

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Abstract

Background: In recent years, several GWAS (genome wide association studies) of sleep-related traits have identified a number of SNPs (single nucleotides polymorphism) but their relationships with symptoms of insomnia are not known. The aim of this study was to investigate whether SNPs, previously reported in association with sleep-related phenotypes, are associated with individual symptoms of insomnia.

Methods: We selected participants from the HUNT study (Norway) who reported at least one symptom of insomnia consisting of sleep onset, maintenance or early morning awakening difficulties, (cases, $N = 2563$) compared to participants who presented no symptoms at all (controls, $N = 3665$). Cases were further divided in seven subgroups according to different combinations of these three symptoms. We used multinomial logistic regressions to test the association among different patterns of symptoms and 59 SNPs identified in past GWAS studies.

Results: Although 16 SNPS were significantly associated ($p < 0.05$) with at least one symptom subgroup, none of the investigated SNPs remained significant after correction for multiple testing using the false discovery rate (FDR) method.

Conclusions: SNPs associated with sleep-related traits do not replicate on any pattern of insomnia symptoms after multiple tests correction. However, correction in this case may be overly conservative.

Keywords: Genetics of insomnia, SNPs, Overlapping phenotypes, Sleep traits, The HUNT study

Background

In recent years, there has been an increasing focus on the genetic basis for sleep/wake traits. Several genome wide association studies (GWAS) have identified numerous single nucleotide polymorphisms (SNPs) that influence sleep traits [1]. For example, Hu and colleagues reported 15 SNPs, several of which were on circadian genes, that were significantly associated with being a "morning person" [2] while a study by Gottlieb et al. focused on sleep duration identified seven SNPs in two circumscribed genetic loci. Caffeine induced insomnia was the object of another GWAS that identified several loci in melatonin and adenosine pathways [3]. Using the same methodology, Byrne et al. identified several loci with plausible biological role influencing sleep quality and timing [4]. Finally, two studies on the UK biobank sample reported more than a hundred novel SNPs associated to accelerometer registered sleep duration, efficiency and number of nocturnal sleep episodes [5, 6] and reproduced three SNPs from previous studies [6].

These sleep-related traits appear to occur with varying frequencies in individuals with insomnia, a condition characterized by decreased quality and/or quantity of sleep in absence of other organic disorders [7]. A study

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by Vgontzas et al. reported that individuals with short sleep duration are almost five times more likely to suffer from persistent insomnia [8]. Several studies have shown that individuals with evening chronotype are more likely to present insomnia symptoms [2, 9, 10], in particular difficulties in falling asleep [11]. Therefore, the co-occurrence of specific sleep traits and insomnia might be the product of a common genetic and biological background.

Several GWAS studies seems to support the hypothesis of pleiotropy. A GWAS study by Stein et al. reported an inverted correlation between genetic loci associated with both insomnia and morning chronotype [12]. Several studies conducted on the UK biobank population presented also overlapping genes for insomnia and sleep duration [13–15]. On the other hand, another GWAS study of the same population did not report any common genes for objective measure of several sleep phenotypes [5].

Most studies on insomnia treat insomnia as a single entity and do not consider individual patterns of insomnia symptoms. Insomnia may presents itself as a combination of night-time symptoms but one of these symptom may prevail over the others: difficulties in falling asleep, trouble with staying asleep and waking up too early. Few studies have examined each individual nocturnal symptom of insomnia, despite evidence that the different symptoms may represent biologically distinct mechanisms. Stoffers et al. described decreased gray matter density in a part of the left orbitofrontal cortex in individuals reporting waking up too early, but not in those reporting trouble with falling asleep or sleep maintenance [16]. Epidemiological studies showed that different symptoms are associated with different incidence of physical and psychiatric conditions [17] and mortality [18]. In one such study, males experiencing sleep onset insomnia or terminal insomnia had a risk three-fold higher than healthy sleepers to receive a disability pension due to a mental condition, while maintenance insomnia gave a considerably lower risk.

For these reasons, we argue that investigating individual insomnia symptoms may aid in the identification of genetic overlap with sleep-related traits. Elucidating the relationship between nocturnal insomnia symptoms and sleep-related traits might clarify the etiology and help diagnostic and therapeutic processes.

In order to investigate this relationship, we conducted an association study on individual symptoms of insomnia and SNPs previously reported to be associated with sleep-related phenotypes. The use of material from the Nord-Trøndelag Health Study (HUNT) gave us the opportunity to investigate this relationship in a large sample from a general population.

Methods

Participants

This study used data from the Nord-Trøndelag Health Study (HUNT3, Norway) performed in 2006–2008. The study is comprised of 50,807 individuals participated in the study providing extensive health information and biological samples. For a detailed overview of all three HUNT cohorts, see [19].

From the total sample we selected 18,606 participants (36.6%) who answered "Never/Seldom" to questions about snoring and interrupted breathing during the night (individuals answering "Sometimes" and "Several times a week" were excluded). Of these, participants with complete data for symptoms of insomnia ($N = 18,473$, 99.3%) were selected. A total of 7933 participants (43%) could be classified as cases or controls. However, analysis of kinship among these excluded 1262 participants, leaving 6281 participants (79%). Genetic data was available for 6029 of these participants. The selection workflow is shown in detail in Fig. 1.

Insomnia

To determine the presence of insomnia symptoms we used three questions contained in the HUNT3 Questionnaire 2 (Sleep section [20]). These three questionnaire items inquire about the frequency of the three core symptoms for insomnia disorder, as specified in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) [7].

"How often in the last 3 months have you:

Had difficulty falling asleep at night?

Woken up repeatedly during the night?

Woken too early and couldn't get back to sleep?"

Possible response options were: "Never/seldom", "Sometimes", "Several times a week".

Answering "Several times a week" to at least one question determined cases ($N = 3577$) while answering "Never/Seldom" to all three questions was used as definition for controls ($N = 2452$).

Cases were further divided in seven subgroups according to the reported pattern of symptoms (Fig. 2).

Genetic data

We selected the SNPs used in this study the consulting the GWAS catalog [21]. This bioinformatics on-line tool "provides a consistent, searchable, visualizable and freely available database of published SNP-trait associations". In 2016, we searched the GWAS catalog using the keywords "insomnia", "sleep" and "chronotype" and selected 52 SNPs from four GWAS studies [3, 4, 22, 23]. Another

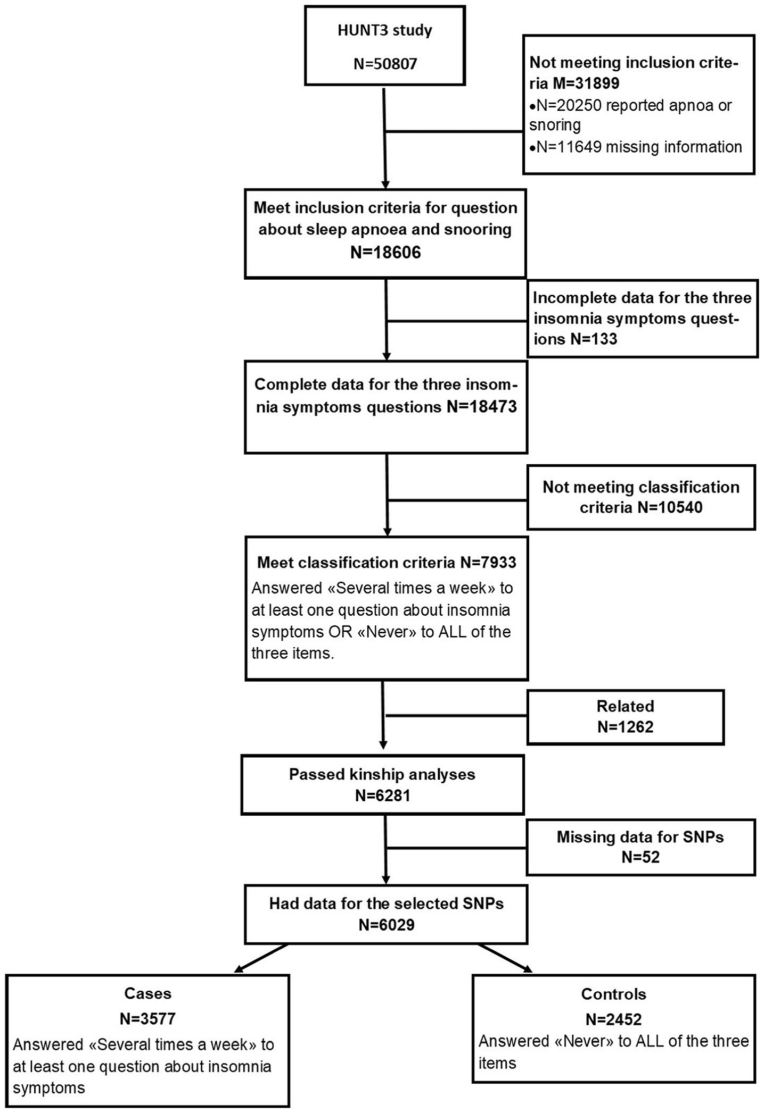

Fig. 1 Selection workflow for the HUNT 3 sample included in this study ($N = 6029$)

fifteen SNPs were included from a GWAS study on "morningness" [2] that was not included in the GWAS catalog at the time of the search. A total of 67 SNPs were included in the study.

Genetic data were obtained from the HUNT databank and the genetic material used is stored by the HUNT biobank.

PLINK (version 1.9) [24] was used to exclude SNPs with a minor allele frequency (MAF) below 5% and those not in Hardy-Weinberg equilibrium (p-value< 0.05).

We excluded participants who were related up to the third degree (kinship coefficient ≥ 0.0884) using FastIndep [25]. After kinship analysis, 1262 individuals were excluded (98 cases and 1164 controls).

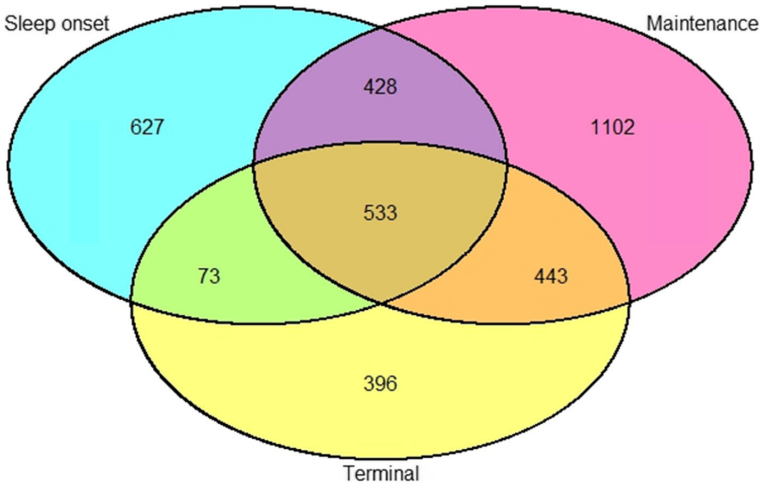

Fig. 2 Distribution of the participants according to reported symptoms of insomnia

Analyses

We used multinomial logistic regression to test the association between the 67 SNPs and the traits (patterns of symptoms of insomnia plus controls as a dependent variable with eight categories). Sex and age in years were included as covariates. Correction for multiple statistical hypotheses was conducted using Benjamini-Hochberg False Discovery Rate (FDR) (58 SNPs × 7 comparisons for a total of 413 tests, alpha = 0.05). All the statistical analyses were conducted using RStudio (Version 1.0.136).

Results

Descriptive statistics

Descriptive statistics are shown in Table 1. The current sample ($N = 6029$) included more females (67%) than males. Mean age for the whole sample was 50, (SD = 16.2, range: 19.2 to 96.8 years), 53 for cases and 45 for controls (t (5545,3) = − 20.55, $p > 0.001$).

Table 1 Descriptive statistics for the sample

	Count	Females (%)	Age (M)
Sleep onset (SOI)	627	71.1	48.9
Maintenance (MI)	1102	72.5	53.0
Terminal (TI)	396	60.6	57.6
SOI + MI	428	80.6	52.1
MI + TI	443	68.6	55.6
SOI + TI	73	78.1	58.7
SOI + MI + TI	533	83.1	55.1
Total in cases	3602	73.1*	53.4*
Controls	2427	58.2	45.2
Total	6029	67.1	50.1

* = $p < 0.05$ for comparison of cases versus controls

Association testing

Nine SNPs with MAF lower than 5% in the CEU population (Northern Europeans from Utah) or our sample were excluded. Therefore, a total of 58 SNP were tested for associations.

Sixteen SNPs presented p-values below 0.05 before correction for multiple testing in eighteen associations (two SNPs were associated with two symptoms subgroups). None of the p-values retained significance after Benjamini-Hochberg FDR correction.

Besides the p-value, the odds ratio for most of the tests could be considered valid. The T allele of rs2302729 on *CACNA1C* (Calcium voltage-gated channel subunit alpha1 C) were 1.9 times more likely than controls ($p = 0.004$, 95% CI [1.2 to 3]) the highest odds ratio for experiencing sleep onset insomnia with terminal insomnia. For the T allele of rs10493596 on gene *AK5* (Adenylate Kinase 5) the same symptom was 66% less likely to occur ($p = 0.004$, OR = 0.4, 95%CI [0.3 to 0.8]) compared to controls. Individuals who reported sleep onset insomnia with maintenance insomnia were 50% more likely than controls to present the T allele of rs113851554 on *MEIS1* ($p = 0.01$, OR = 1.5, 95% CI [1.1 to 2]). Annotations for all 16 SNPs are collected in Table 2. Results for all SNPs are presented in Additional file 1.

Discussion

Sixteen SNPs previously associated with sleep-related traits were significantly associated with at least one symptom or a

Table 2 SNPs showing significant p-value before FDR correction

SNP	Ref. allele	Other allele	Gene	Symptoms sub-group	B	OR	95% CI	*p*-value	Previous associations
rs10493596	T	C	*AK5*	SOI + TI	−0.82	0.45	[0.3 to 0.8]	0.004	Morning chronotype [2]
rs10823607	T	C	*ADAMTS14*	SOI + TI	− 0.71	0.5	[0.2 to 0.9]	0.039	Sleep duration [4]
rs113851554	T	G	*MEIS1*	SOI + MI	0.4	1.5	[1.1 to 2]	0.007	Insomnia symptoms [13]
				SOI + MI + TI	0.36	1.4	[1.1 to 2]	0.007	
rs11706236	G	A	*CACNA2D3*	MI + TI	−0.33	0.7	[0.5 to 0.9]	0.009	Caffeine related insomnia [3]
rs12471454	T	C	*SATB2*	TI	−0.26	0.8	[0.6 to 1]	0.029	Insomnia [4]
rs12927162	G	A	*TOX3*	MI	−0.15	0.9	[0.7 to 1]	0.036	Morning chronotype [2]
rs1823125	G	A	*PAX8*	MI	0.16	1.2	[1 to 1.4]	0.033	Sleep duration [23]
rs1940013	T	C	*OPCML*	SOI	0.19	1.2	[1 to 1.5]	0.037	Usual bedtime [22]
rs2221285	T	C	*ESRRG*	SOI + TI	−0.53	0.8	[0.7 to 1]	0.027	Sleep duration [23]
rs2287838	G	A	*PIN1*	SOI	−0.22	0.8	[0.7 to 1]	0.022	Sleep duration [23]
rs2302729	T	C	*CACNA1C*	SOI + TI	0.64	1.9	[1.2 to 3]	0.009	Sleep latency [4]
rs34714364	T	G	*APH1A*	MI	0.16	1.18	[1 to 1.4]	0.041	Morning chronotype [2]
rs55694368	T	G	*PER2*	MI	−0.19	0.83	[0.7 to 1]	0.043	Morning chronotype [2]
rs6437122	G	C	*UPP2*	SOI + MI + TI	−0.34	0.7	[0.5 to 0.9]	0.015	Sleep duration [23]
rs9517132	T	C	*RANBP5*	SOI	−0.22	0.8	[0.7 to 1]	0.018	Usual sleep duration [23]
rs9804200	C	T	*EBF3*	MI + TI	−0.21	0.81	[0.7 to 1]	0.044	Usual bedtime [4]

SOI sleep onset insomnia, *MI* maintenance insomnia, *TI* terminal insomnia

combination of symptoms of insomnia. However, none of these variations stayed significant after correction for multiple statistical testing.

Among our highest hits, there was SNP rs10493596. This variation is close to the *AK5* (Adenylate Kinase 5) gene that encodes for an adenylate kinase expressed exclusively in the brain. This protein is involved in ATP homeostasis by catalyzing the transfer of phosphate groups among adenine nucleotides. Rs10493596 was associated with "morningness" in a study by Hu et al. [2] while in our study it gave the lowest *p*-value for difficulties falling asleep in combination with early morning awakenings.

Difficulties in falling asleep with early morning awakenings showed also an association with rs2302729 that was previously associated with sleep quality and latency [4]. This SNP is located on *CACNA1C* (Calcium Voltage-Gated Channel Subunit Alpha1 C) whose involvement in several psychiatric conditions is supported by epidemiological and animal studies [26]. Knockout mice for *CACNA1C* display traits that resemble symptoms of mental disorders and autism such as cognitive decline, anxiety, hyperactivity, decreased sociability, decreased synaptic plasticity [27].

Of note is the presence of a polymorphism on gene *MEIS1* among our highest hits. *MEIS1* is involved in restless leg syndrome (RLS) [28] but recently also reported to be associated with insomnia symptoms [5, 13, 29]. In our study, combinations of symptoms sleep onset problems with maintenance insomnia and all symptoms together showed low *p*-values (0.01 and 0.02 respectively) and discrete odds ratio (1.5 and 1.4) for the T allele of rs113851554, in agreement with previous studies. This finding strengthens the hypothesis that insomnia and RLS may be overlapping phenotypes not easy to discern [13].

The A allele of rs12927162 on gene*TOX3* (TOX High Mobility Group Box Family Member 3), decreased the chances of maintenance insomnia. This SNP is reported in significant association with being a morning person [2] but also with measure of circadian phase delay [5].

Rs1823125 near gene *PAX8* was firstly reported as associated with sleep duration in the CHANGE consortium sample [23], and successively in the UK Biobank sample [6] in which it was associated also with sleep efficiency [5]. In our study, it was associated with maintenance insomnia. PAX8 is a transcription factor with proven role in kidney and thyroid morphogenesis. Its role on sleep is yet to be investigated, and it is possible that rs1823125 is not in fact influencing *PAX8* but another gene nearby as it is located in a intragenic region.

Most of our results seems to have a plausible explanation in spite of ending up statistically non-significant after correction for multiple testing. The need for correction when testing multiple hypotheses may often be redundant especially in the context of biology or in K. J. Rothman words "… when scientists are studying biological relations rather than random numbers, the premise that type I errors are the major concern may be wrong" [30]. Therefore overestimating the role of "chance" when analyzing biological data

may lead to type II errors. In our case, we chose SNPs known to influence sleep-related traits, therefore their involvement in insomnia is plausible.

Strengths and limitations

The HUNT study collected data from Norwegians from the region of Nord-Trøndelag which gives the advantage of a relative-high genetic isolation and exposure to similar environmental factor that may influence sleep (natural light, cultural habits etc.). Also, strong welfare polices implemented in Norway lessen the effect of socioeconomic disparities that may affect the analyses.

Unfortunately, the HUNT study did not gather information about sleep length or satisfaction that could have helped determining the presence of an actual disorder. Inclusion of this information in future studies and the finding of endophenotypes may help the discovery of relevant genetic associations.

Several of our significant SNPs (before correction for multiple testing) were associated with the subgroup of symptoms "sleep onset with early morning awakenings". This combination of symptoms was the rarest, with only 73 individuals reporting it. This further support the strength of the association.

Conclusions

After multiple testing correction, we did not find any statistically significant association between combination of symptoms of insomnia and several SNPs associated with sleep-related phenotypes. However, the presence of a biological explanations and early reports on similar phenotypes makes vigorous use of correction for multiple statistical testing questionable.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12881-019-0916-6.

Additional file 1. Full results for the multinomial regression analysis.

Abbreviations

ADAMTS14: ADAM Metallopeptidase with Thrombospondin Type 1 Motif 14; *AK5*: Adenylate Kinase 5; *APH1A*: Aph-1 Homolog A, Gamma-Secretase Subunit; ATP: Adenosine triphosphate; *CACNA1C*: Calcium voltage-gated channel subunit alpha1 C; *CACNA2D3*: Calcium voltage-gated channel auxiliary subunit alpha 2 Delta 3; CEU: Utah Residents (CEPH) with Northern and Western European Ancestry; DSM-5: Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition; *EBF3*: EBF Transcription Factor 3; *ESRRG*: Estrogen Related Receptor Gamma; FDR: False discovery rate; GWAS: Genome wide association study; HUNT: Helse Undersøkelse Nørd-Trøndelag (Nord-Trøndelag Health Study); MAF: Minor allele frequency; *MEIS1*: Meis Homeobox 1; *OPCML*: Opioid Binding Protein/Cell Adhesion Molecule Like; *PAX8*: Paired Box 8; *PER2*: Period Circadian Regulator 2; *PIN1*: Peptidylprolyl Cis/Trans Isomerase, NIMA-Interacting 1; *RANBP5*: Uridine Phosphorylase 2; *SATB2*: SATB Homeobox 2; SNPs: Single nucleotide polymorphisms; *TOX3*: TOX High Mobility Group Box Family Member 3; *UPP2*: Uridine Phosphorylase 2

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Authors' contributions

DB: selection, analysis of the genetic data and draft of the manuscript; BS: supervision of the study; PG: critical reading and discussion of manuscript; SL: design and interpretation of the statistical methodology and results; ICG: design and supervision of the study; All authors: critical revision of the manuscript and contribution to the intellectual content of the work. All authors read and approved the final manuscript.

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Availability of data and materials

Data used in this study is available on request to the HUNT databank (https://hunt-db.medisin.ntnu.no/hunt-db/#/) and is subject to fees as decided by the HUNT Research Centre (https://www.ntnu.edu/hunt/data). The authors of the present study do not have permission to share the dataset obtained from HUNT.

Ethics approval and consent to participate

This study was approved by the Regional Committee for Medical and Health Research Ethics of South-East Norway (reference number 2016/672) on date 04.27.2016. All participants in the HUNT study signed a written informed consent form allowing the use of their data and samples for research. Participants can demand to have their data deleted from the HUNT database at any given moment.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Differences in anxiety levels among symptoms of insomnia. The HUNT study

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ABSTRACT

Objectives: This study aim is to compare anxiety levels among individuals experiencing different symptoms of insomnia.
Design: Case-control study.
Setting: The Nord-Trøndelag Health Study (the HUNT3 study, Norway).
Participants: Of the 50,802 individuals taking part in the HUNT3 study, the current sample comprised 7933 individuals, including 4317 cases with insomnia and 3616 controls.
Measurements: Symptoms of anxiety were assessed using Hospital Anxiety and Depression Scale, whereas insomnia symptoms were assessed according to the core *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition,* nocturnal symptoms. Anxiety levels of the 4317 individuals reporting at least 1 insomnia symptom were compared with the 3616 controls reporting no symptoms. Level of anxiety among participants experiencing combinations of insomnia symptoms was also investigated.
Results: Anxiety levels were significantly higher in individuals reporting insomnia symptoms ($M = 2.5$, SD = 2.4) compared to controls (5.5, SD = 3.7, $P < .001$). Anxiety levels also differed significantly between different insomnia symptoms ($P < .001$). Participants reporting all 3 insomnia symptoms had the highest anxiety score ($M = 6.8$, SD = 4.3), followed in decreasing order by sleep onset insomnia with terminal insomnia ($M = 6.7$, SD = 4.0), sleep onset insomnia with sleep maintenance insomnia ($M = 6.3$, SD = 3.8), sleep onset insomnia only ($M = 5.8$, SD = 3.7), sleep maintenance insomnia with terminal insomnia ($M = 5.6$, SD = SD = 3.4), terminal insomnia ($M = 5.2$, SD = 3.4), and sleep maintenance insomnia only ($M = 4.5$, SD = 3).
Conclusions: Difficulties initiating sleep, both alone and in combination with 1 or 2 of the other symptoms, seem to play a key role in rising anxiety levels.

Introduction

Insomnia and anxiety are interlinked throughout the lifespan,[1] with 20%-30% of people with insomnia also presenting with significant anxiety. Insomnia and anxiety do not merely co-occur; they also seem to influence each other over time, and there is increasing evidence that the relationship between insomnia and anxiety is most likely bidirectional.[2,3] The 2 conditions are hypothesized to be either different expressions of the same response to psychological distress, or distinct conditions with shared symptoms or other underlying common factors.[3]

From a biological point of view, the co-occurrence of insomnia and anxiety may be caused by dysregulation of specific corticolimbic circuits responsible for both emotional responses and sleep.[4] Genetic studies support[5] this theory, reporting common predisposing genetic variations for these conditions. The corticotrophin-releasing hormone system is another biological element wiring insomnia and

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anxiety, which is involved in promoting psychological arousal. In a state of anxiety, catecholamines are released, resulting in psychological and physiological alertness and hence anxiety and insomnia.[6]

An often overlooked aspect of insomnia is that people with the disorder may present with 1 symptom only or a combination of several. According to the *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition* (DSM-5),[7] there are 3 principal nocturnal symptoms of insomnia: difficulties in falling asleep (or sleep onset insomnia), difficulty staying asleep (or maintenance insomnia), and terminal insomnia (terminal insomnia). Although most researchers treat these symptoms equivalently or combine them to produce a joint operationalization of an insomnia disorder, few studies have examined each insomnia symptom individually in this context.

The established relationship between insomnia and anxiety is based on studies that do not consider possible differences in the symptoms. Comparing different symptoms with regard to their consequences and background is necessary to validate the customary treatment of these symptoms as equal. In one such study, Canivet and colleagues brought evidence of a different risk of somatic and mental disorders among the aforementioned insomnia symptoms.[8] For example, men reporting sleep onset insomnia or terminal insomnia were 3 times more likely than healthy sleepers to receive a disability pension due to mental problems, whereas maintenance insomnia gave a considerably lower risk. These findings suggest that different insomnia symptoms may have different implications and consequences, including different patterns of anxiety. Few studies[9–11] have reported anxiety levels for single symptoms of insomnia, but small sample size and low resolution in defining the 3 symptoms and their combinations call for further investigations.

The overall aim of this study was to report and compare anxiety levels among individuals reporting different symptoms of insomnia using a large population-based sample, the Nord-Trøndelag Health Study (HUNT). This large dataset gives the unique opportunity to investigate this very relationship, as it has collected data from inhabitants on both insomnia symptoms and anxiety.

Methods

Participants

This study is part of the Nord-Trøndelag Health Study (the HUNT study, Norway) that includes data from 3 cohorts. In the present

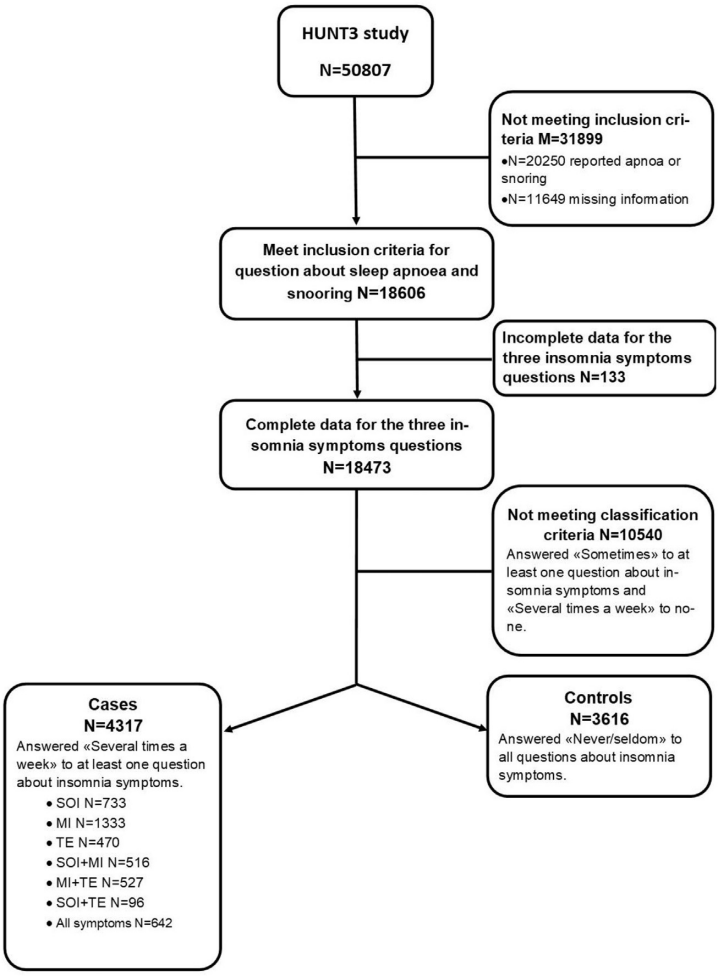

Fig. 1. Study design and participant flow of the study sample. SOI, sleep onset insomnia; MI, maintenance insomnia; TE, terminal insomnia.

study, we used data from the HUNT3 cohort (2006-2008) which comprised 50 807 participants. As with the first 2 HUNT cohorts, in HUNT3, all citizens (N = 93 860) from the Norwegian region of Nord-Trøndelag aged 20 years or more were invited to provide extensive health information and biological samples. Detailed information about the HUNT studies has been summarized by Krokstad et al.[12]

Figure 1 shows the design and participant flow of the current sample. Of 50 807 participants, 50 802 individuals provided valid responses on the relevant HUNT3 questionnaires. Of these, we selected 18 606 individuals (36.6%) who answered "Never/Seldom" to questions regarding the frequency of snoring or interrupted breathing during the night (ie, possible proxy for sleep apnea) during the past month. Of these, participants with complete data for all 3 symptoms of insomnia (n = 18 473, 97.3%) were selected and comprised the current study sample.

Insomnia

Three questions in the HUNT3 Questionnaire 2 (Sleep section,[13] the 3 items can be found in section S1 of Supplementary material) cover the principal symptoms of insomnia as stated in the DSM-5[7]: difficulties falling asleep (sleep onset insomnia), woken up repeatedly during the night (maintenance insomnia), and woken too early and could not get back to sleep (terminal insomnia). These questions investigate the frequency of the symptoms during the previous 3 months using the following response options: "Never/seldom," "Sometimes," and "Several times a week."

Study participants were classified into insomnia cases and controls. Those who answered "Several times a week" to at least 1 symptom were classified as cases (n = 4317), and those who answered "Never/Seldom" to all 3 questions constituted the controls (n = 3616). The rest of the respondents answering "Sometimes" to at least 1 question and "Several times a week" to none of them were excluded. Cases were further assigned to 1 of 7 subgroups, according to the combinations of the reported insomnia symptoms.

Anxiety measure

The Hospital Anxiety and Depression Scale (HADS)[14] is a 14-item questionnaire used to evaluate symptoms of depression and anxiety and is a popular tool among both clinicians and researchers.[15] In this study, we used the sum of the 7 items related to anxiety (HADS-A), giving a sum score in the range from 0 to 21.

Analyses

A total of 493 individuals (2.7%) in our working dataset lacked data on 1 or more HADS-A items. We used single imputation (expectation-maximization algorithm) to singly impute these missing data. The imputation model included the variables age and HADS-A items.

Student *t* test was used to compare HADS-A scores between cases and controls. We used linear regression with HADS-A as dependent variable and type of sleep symptoms as an 8-category covariate, adjusting for sex and age. Differences in HADS-A levels among cases in subgroups were compared using Bonferroni adjustment accounting for all the pairwise comparison between the 7 groups. Separate analyses for men and women were conducted in the same way. Because the residuals were not normally distributed, we used bootstrapping with 1000 bootstrap samples.

Separately, participants were classified according to HADS-A score into 4 groups by increasing severity: normal (0-7), mild (8-10), moderate (11-14), and severe (15-21), according to the earlier published classification criteria.[14] Frequencies of the insomnia symptoms and their combination were examined for each group.

We compared HADS-A scores and age between cases, controls, and excluded individuals using analysis of variance with 1000 bootstrap samples. A χ^2 test was used to examine differences in distribution of sexes among the 3 groups.

All analyses were conducted using IBM SPSS 25 (SPSS Inc, Chicago, IL).

Results

Overall, our sample (n= 7933) included more women (66.2%) than men. Sixty-four percent of women were classified as insomnia cases compared to 48.2% of men (χ^1 =144, 6, $P < .001$). Mean age for the sample was 50.2 years (SD = 16.2, range: 19.2-96.8). Cases were significantly older ($M = 54$) than controls ($M = 45$) (t[7931] =26.29, $P < .001$). As detailed in Table 1, the level of anxiety was significantly higher (t[7931] =41.65, $P < .001$, mean difference = 3) in cases ($M = 2.5$, SD = 2.4) than controls ($M = 5.5$, SD = 3.7).

HADS-A levels were significantly higher in women for both cases and controls. When analyzing single symptoms separately, anxiety levels were significantly higher in women experiencing from sleep onset insomnia (t[731] =1.9, P= .049) and maintenance insomnia (t[761.2] =4.6, $P < .001$).

The regression results showed that the level of anxiety differed significantly among the types of insomnia symptoms after controlling for sex and age (F[6, 4317] = 43.92, $P < .001$). Table 2 shows the results for Bonferroni-corrected group comparisons. Participants reporting all 3 insomnia symptoms had the highest anxiety score ($M = 6.8$, SD = 4.3), followed in decreasing order by sleep onset insomnia with terminal insomnia ($M = 6.7$, SD = 4.0), sleep onset insomnia with sleep maintenance insomnia ($M = 6.3$, SD = 3.8), sleep onset insomnia only ($M = 5.8$, SD = 3.7), sleep maintenance insomnia with terminal insomnia ($M = 5.6$, SD = 3.4), terminal insomnia ($M = 5.2$, SD = 3.4), and sleep maintenance insomnia only (M = 4.5, SD = 3). Mean anxiety score for maintenance insomnia differed significantly from all other symptoms except terminal insomnia

Table 1
Descriptive statistics by subgroups of symptoms of insomnia.

		Sleep onset (SOI)	Maintenance (MI)	Terminal (TI)	SOI + MI	MI + TI	SOI+ TI	All symptoms	Tot. cases	Controls	Total
n		733	1333	470	516	527	96	642	4317	3616	7933
Female %		72	71.6	62.1	80.8	69.6	80.2	81.9	73.2*	57.9	66.2
Age (y), *M* (SD)		49.2	53.9	57.2	52.4	56.1	58.3	55.4	53.9	45.1	50.2
		(17.8)	(16.3)	(15.2)	(15.3)	(14.2)	(18.1)	(15.4)	(16.2)*	(14.7)	(16.2)
HADS-A M (SD)	Tot.	5.9	4.5	5.2	6.3	5.7	6.5	7	5.6	2.5	4.3
		(3.8)	(3.3)	(3.4)	(3.8)	(3.4)	(3.5)	(4.4)	(3.8)*	(2.4)	(3.6)
	Female	6.0***	4.7**	5	6.4	5.7	6.6	7	5.7	3.9**	4.7**
	Male	5.4	3.9	5.2	5.9	5.6	7.4	6.3	5.1	2.8	3.9

* *P* value < .001 for case-control comparison.
** *P* value < .001 for male-female comparison.
*** *P* value = .049 for male-female comparison.

Table 2
Results for Bonferroni-corrected pairwise comparisons of symptoms of insomnia subgroups for HADS-A scores

Pairwise comparisons		MD	*P* value	95% CI
Onset	Maintenance*	1.3	<.001	[1 to 1.6]
	Terminal*	0.7	.029	[0.2 to 1.1]
	Onset + maintenance	−0.5	.518	[−0.9 to −0.1]
	Maintenance + terminal	0.1	1.0	[−0.2 to 0.5]
	Onset + terminal	−1.0	.324	[−1.9 to −0.1]
	All symptoms*	−1.0	<.001	[−1.5 to −0.6]
Maintenance	Terminal*	−0.6	.028	[−1 to −0.3]
	Onset + maintenance*	−1.8	<.001	[−2.1 to −1.4]
	Maintenance + terminal*	−1.2	<.001	[−1.5 to −0.8]
	Onset + terminal*	−2.3	<.001	[−3.2 to −1.4]
	All symptoms*	−2.4	<.001	[−2.7 to −2]
Terminal	Onset + maintenance*	−1.2	<.001	[−1.6 to −0.7]
	Maintenance + terminal	−0.5	.397	[−1 to −0.1]
	Onset + terminal*	−1.6	.001	[−2.5 to −0.8]
	All symptoms*	−1.7	<.001	[−2.2 to −1.3]
Onset + maintenance	Maintenance + terminal	0.6	.133	[0 to 2.1]
	Onset + terminal	−0.5	1.000	[−1.4 to 0.5]
	All symptoms	−0.6	.149	[−1.1 to −0.1]
Maintenance + terminal	Onset + terminal	−1.1	.133	[−2.1 to −0.2]
	All symptoms*	−1.2	<.001	[−1.6 to −0.8]
Onset + terminal	All symptoms	−0.1	1.000	[−0.9 to 0.8]

Differences in mean values can be considered as effect measure for the pairwise comparisons.[24] MD, mean difference.
* Significant results.

only (Table 2). Other groups significantly different from one another are reported in Table 2.

Results from pairwise comparison of mean anxiety level among insomnia subgroups differed in some cases between men and women (Table S2 of Supplementary Material).

The distribution of insomnia symptoms according to the 4 HADS-A levels is reported in Figure 2.

Analysis of variance results for differences in HADS-A levels among cases, controls, and individuals excluded from the study were significant ($F[2, 18\,470] = 1055.45$, $P < .001$). Post hoc analysis showed that HADS-A mean score for each group significantly differed from one another, with excluded individuals ($M = 3.6$) lying between controls ($M = 2.4$) and cases ($M = 5.5$). Age followed a different trend ($F[2, 18470] = 367.97$, $P < .001$) with excluded participants presenting the highest mean age ($M = 54$), followed by cases ($M = 52$) and controls ($M = 45$). Finally, excluded participants showed a percentage of women significantly higher (73.2%) than the others groups (66.3% in cases and 58% in controls), $\chi^2 = 207.2$, $P < .001$.

Discussion

The aim of the current study was to investigate the level of anxiety across individual and combinations of symptoms of insomnia using data from a large population-based sample. In short, anxiety levels were higher in participants reporting symptoms of insomnia compared to controls. Moreover, anxiety levels differed among symptoms and their combinations.

First of all, we found anxiety levels to be significantly higher in cases than controls. This is in agreement with a systematic review of the literature conducted by Cox et al,[1] who also reported a higher frequency of sleep disturbances among people affected by

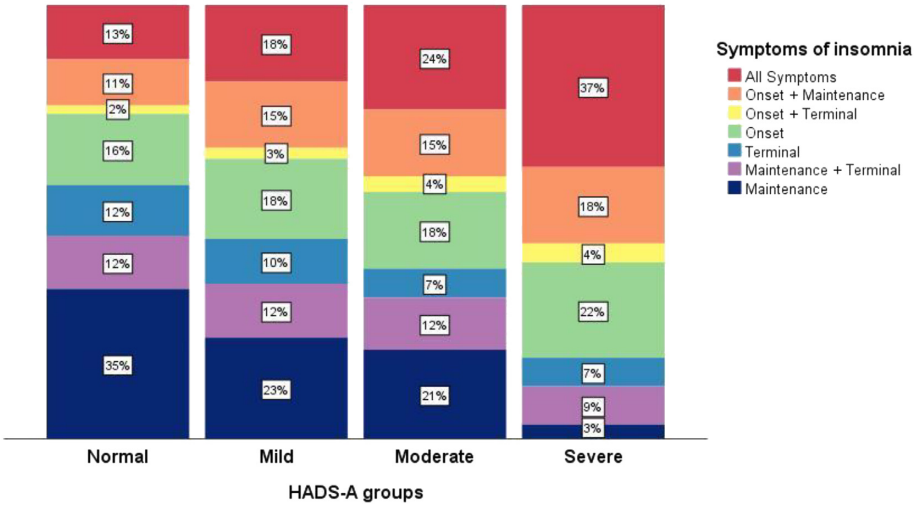

Fig. 2. Distribution of symptoms of insomnia among levels of HADS-A. Normal (0-7), mild (8-10), moderate (11-14), and severe (15-21).

generalized anxiety disorder, panic disorder, and posttraumatic stress disorder than healthy controls.

In our sample, anxiety was significantly lower for maintenance insomnia than terminal insomnia by 0.7 point ($P = .029$) and sleep onset insomnia by 1.4 points ($P < .001$). Studies suggests that the minimal important difference for HADS-A is 1.5 to 2.5[16,17] points; therefore, the difference in anxiety between maintenance and sleep onset insomnia could be considered of borderline importance. To our knowledge, only 3 comparable studies reported anxiety in insomnia symptoms earlier. Cervena et al compared electroencephalographic spectra of people with problems in sleep onset or sleep maintenance and good sleepers and reported mean HADS scores.[9] Taylor et al compared onset, maintenance, and mixed insomnia for demographic and health-related factors including anxiety levels using the State Trait Anxiety Inventory.[10] Finally, a study by Pillai et al[11] compared results of the Beck Anxiety Inventory between subjects reporting sleep onset insomnia and sleep maintenance insomnia. In contrast to ours, none of these studies found a significant difference between individual symptoms of insomnia. These differences might be attributed to the low number of participants in the studies of Cervena et al and Taylor et al ($N = 30$ and $N = 149$) and, generally, to the use of different measurement tools for anxiety and inclusion criteria for the participants.

The differences in HADS-A score we observed could be an expression of unequal levels of stress associated with different symptoms of insomnia. Vgontzas and colleagues[18] proposed that objectively shortened sleep is a more severe insomnia subtype compared to subjective shortened sleep because affected individuals experience both psychological and physiological distress. Difficulties in falling asleep may reduce sleep length, whereas very short nocturnal awakenings may not interfere excessively with the amount of sleep and consequently may not create comparable distress. However, our questionnaire did not investigate the length of these nocturnal awakenings, and therefore, these arguments remain speculative.

In our study, participants experiencing any combinations of 2 symptoms concomitantly showed similar anxiety levels with individuals experiencing sleep onset insomnia alone. Moreover, participants reporting sleep onset insomnia alone or in combination with other symptoms constituted 91% of those who were classified as experiencing severe anxiety according to HADS-A (Fig. 2). This suggests that sleep onset insomnia alone is as severe in terms of anxiety as experiencing several symptoms of insomnia at the same time. This is in line with another finding reported in the aforementioned study by Pillai et al that concomitant sleep onset and maintenance symptoms presented significantly higher anxiety scores than single symptoms.[11]

Participants reporting all 3 symptoms of insomnia had the highest mean HADS-A score, but this was not significantly different from combinations of sleep onset with another symptom. Mean HADS-A score for those who were experiencing all 3 symptoms concomitantly was 4.5 points higher than controls, a whole 21% of the full scale. Subjects reporting all 3 symptoms scored also 2.7 points higher than maintenance insomnia, which is 11.4% of the full scale and the highest difference among the symptoms subgroups. Sleep disturbances appear as symptoms of several severe anxiety disorders, and studies showed that, often, both initiation and consolidation of sleep are affected simultaneously in these conditions.[1] Although HADS is not meant as a diagnostic tool and our results should be interpreted primarily as basic research, the measure of these effects exceeding the reported minimal important difference values points to the possible clinical importance of these findings.

In this study, we find differences in HADS-A levels between men and women presenting maintenance insomnia and sleep onset insomnia. Further studies including sex-specific factors are necessary to clarify the nature of these differences.

In terms of clinical importance, these findings suggest that current therapeutic approaches need further definition and personalization. Several studies have examined to what extent monotherapeutic interventions designed specifically for insomnia or anxiety may improve symptoms of the other disorder. However, a meta-analysis from Belleville and colleagues showed that cognitive behavioral therapy for insomnia only had moderate impact on anxiety levels among insomnia patients and that administration of cognitive behavioral therapy for anxiety alone had limited effects on improving insomnia symptoms.[19] This suggests that a more fruitful approach may be to combine interventions for each condition into a more comprehensive treatment approach. For example, research has shown that anxiety management training aimed at reducing bodily tension has beneficial effects on sleep onset insomnia specifically.[20] As such, a closer characterization of the relationship between insomnia symptoms and anxiety might benefit the design of personalized, more effective therapeutic solutions.

Strengths and limitations

Using data from a large population cohort allowed us to include a high number of individuals for each combination of symptoms. Participants in the HUNT study are Norwegian citizens who all reside in the region of Nord-Trøndelag. This granted us genetic and relative environmental homogeneity in the population. Moreover, uniformity in the Norwegian society in terms of socioeconomic status and health care accessibility reduced the confounder potential of these factors.

The HUNT3 study contained only the 3 aforementioned questions on insomnia. Lack of information about sleep length, sleep satisfaction, and duration of the sleep problems did not allow us to refine the characterization of insomnia any further.

The prevalence of insomnia in the HUNT study,[21] the source of our study sample, was similar to other studies that used similar criteria.[22,23] However, our special selection criteria regarding the absence of snoring, interrupted breathing, and how frequent the symptoms occurred make it difficult to compare the frequency of symptoms in our study to other population studies.

As mentioned before, the effect measure of our results suggests a possible clinical relevancy in these findings. However, the use of HADS as a measure of anxiety calls for careful interpretation.

Conclusions

Different symptoms and combinations of symptoms of insomnia are associated with different levels of anxiety. People with difficulties in falling asleep had the highest anxiety levels both alone and in combination with 1 or 2 of the other symptoms. On the contrary, people with maintenance insomnia had the lowest anxiety levels. We believe investigating this relationship between the principal symptoms of insomnia and anxiety may help refine therapeutic approaches not only for anxiety but also for insomnia.

Conflict of interest

The authors have no conflicts of interest to disclose.

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Council, Central Norway Health Authority, and the Norwegian Institute of Public Health.

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Paper 4

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Epidemiological differences in levels of depressive signs among nocturnal symptoms of insomnia; results from the HUNT study

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Abstract

Background: Insomnia is a sleep disorder characterized by multiple nocturnal symptoms (sleep onset, maintenance and terminal insomnia). However, these symptoms are assumed to have the same weight in the diagnosis and consequences of insomnia. In particular, little is known regarding whether these nocturnal symptoms are equally related to depression. In this study, we compared level of depressive signs among individuals reporting different patterns of nocturnal symptoms of insomnia.

Methods: We used data from the large population-based HUNT3 study. The final sample included 7933 individuals (4317 cases, 3616 controls). Signs of depression were measured using the depression subscale of the Hospital Anxiety and Depression Scale (HADS-D), while the three nocturnal symptoms of insomnia were assessed using a Likert-like scale ("Never", "Sometimes", "Several times a week"). Individuals reporting to experience at least one symptom of insomnia "Several times a week" were grouped according to their pattern of reported symptoms and their HADS-D levels compared.

Results: Participants reporting sleep onset insomnia combined with terminal insomnia had the highest depression score (M = 5.4, SD = 3.4), but reporting maintenance insomnia in addition does not increase the HADS-D scores any further (M = 5.2, SD = 3.6). Accordingly, sleep maintenance insomnia alone had the lowest score (M = 3.4, SD = 2.9).

Conclusions: We found several differences among patterns of symptoms of insomnia but not all of them are clinically relevant. Further studies in clinical samples may help reveal relevant differences among patterns of symptoms, which may aid in refining interventions for concomitant depression and insomnia.

Keywords: HUNT study, Insomnia, Depression, Sleep onset insomnia, Maintenance insomnia, Early morning awakenings

Introduction

According to the fifth edition of Diagnostic and Statistical Manual of psychiatric diseases, (DSM-5) (American Psychiatric Association, 2013), the night-time symptoms of insomnia are difficulties initiating sleep (sleep onset insomnia), several awakenings during the night (maintenance insomnia) and early morning awakenings (terminal insomnia). These three symptoms are weighed equally in clinical diagnosis and few published studies report only scarce characterization of the three symptoms rather than focusing on the validity of this assumption. This may lead to the understanding that different symptoms of insomnia should not differ in their relationship to ills that are commonly coexisting with insomnia, such as depression or anxiety, as well as to social and economic consequences.

As most studies in the field of insomnia research focuses on the final clinical diagnosis, detailed analysis of findings according to the different symptoms is rare.

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Considered the complexity of the possible combinations of symptoms, published research does not make it easier to evaluate the symptoms individually. In order to have a better understanding of the individual symptoms and their significance in the diagnosis, more research on the characterization of symptoms of insomnia and their consequences is required.

Although few, some studies investigated how these symptoms might have different implications and consequences. Previous studies have shown how individuals experiencing trouble with sleep onset were about three times more likely than good sleepers to receive a disability pension due to a mental condition. This risk was higher than for other nocturnal symptoms of insomnia (Canivet et al., 2014). In a recent study we reported that difficulties initiating sleep alone plays a leading role in rising anxiety levels and different combinations of symptoms concur with different strengths of anxiety symptoms (Bragantini et al., 2019). Moreover, magnetic resonance imaging (MRI) studies seems to support biological differences for individual insomnia symptoms, as orbitofrontal grey matter appeared reduced only in patients experiencing early morning awakenings (Stoffers et al., 2012). The DSM-5 report specifically this nocturnal symptom of insomnia among the diagnostic criteria for melancholic depression and indeed patients with comorbid depression and insomnia show also reduced grey matter in the OFC (Yu et al., 2018).

As in case of anxiety, individuals experiencing insomnia are more likely to present depressive symptoms and develop depression (Alvaro et al., 2013; Lichstein et al., 2017). Several studies have demonstrated that the relationship between the two conditions is likely bi-directional (Jansson-Frojmark & Lindblom, 2008; Sivertsen et al., 2012), but some studies report insomnia as increasing the risk for depression but not the opposite (Johnson et al., 2006). Evidence regarding depression and its relationship with individual nocturnal symptoms of insomnia is conflicting. Cervena et al. reported higher levels of depression in individuals experiencing sleep maintenance problems in comparison to good sleepers (Cervena et al., 2014), whereas this was not the case for other insomnia symptoms. In contrast, experiencing sleep onset problems in combination with sleep maintenance problems was associated with higher depression levels in another study (Taylor et al., 2005). Conversely, a third study found no differences in depression severity between individuals experiencing sleep onset and maintenance problems (Pillai et al., 2015). However, these studies had some methodological shortcoming: 1) they did not investigate different patterns/combinations of the nocturnal symptoms of insomnia; and 2) they had generally small study samples.

Identifying differences in severity of depressive symptoms among patterns of symptoms of insomnia in a large cohort may improve our understanding of the interrelationships between insomnia and depression and help refining therapeutic interventions. Based on these considerations, the aim of this study was to assess the level of self-reported depressive signs in subjects reporting also nocturnal symptoms of insomnia, considering all existing patterns and using data from a general population, the HUNT 3 cohort.

Examining the relationship among the individual nocturnal symptoms of insomnia and depressive symptoms more closely may promote our understanding of symptoms of insomnia. This will help evaluate the weight of individual symptoms to the diagnosis and burden of insomnia.

Methods

The individuals in this study were participants in the Nord-Trøndelag Health Study (the HUNT study, Norway). The present study is based on data from 50, 807 citizens collected during the third cohort (2006–08). All citizens ($N = 93,860$) from the region of Nord-Trøndelag, in Norway who were at least 20 years old were asked to complete a questionnaire providing health information and biological samples. Krokstad et al. (Krokstad et al., 2013) summarized in detail all information about the HUNT study.

The selection work-flow of this study can be found here (Bragantini et al., 2019). Of the total HUNT 3 participants, five individuals had no valid answers for the variables relevant for this study. Of the remaining 50,802 those who answered "Never/Seldom" ($N = 18,606$, 36.6%) to questionnaire items regarding the frequency of snoring or interrupted breathing during the sleep (i.e. possible proxy for sleep apnoea) during the past month were selected. A total of 18,473 (97.3%) participants presented complete data for all three symptoms of insomnia and were selected to form the sample investigated in this study.

Nocturnal symptoms of insomnia

The frequency of occurrence of the three nocturnal symptoms of insomnia were determined using participant's answers to the Questionnaire 2 (Sleep section) of the HUNT 3 study:

"How often in the last 3 months have you:

Had difficulty falling asleep at night?

Woken up repeatedly during the night?

Woken too early and couldn't get back to sleep?"

"Never/seldom", "Sometimes", "Several times a week" were the possible response options.

Participant who reported to experience at least one symptoms with a frequency of "Several times a week", were classified as cases ($N = 4317$). Participants who answered "Never/seldom" to all questions were defined as controls ($N = 3616$). Answering "Sometimes" to at least one question and "Several times a week" to none of them determined the exclusion from the study. Cases were further divided according to the pattern of symptoms they presented into seven subgroups.

Measure of depressive signs

The Hospital Anxiety and Depression Scale (HADS) (Zigmond & Snaith, 1983) is a validated (Bjelland et al., 2002) 14-items questionnaire used to evaluate symptoms of depression and anxiety. Here, we used the sum of the seven items related to depression (HADS-D), giving a score in the range from zero to 21. Cronbach's alpha coefficient of internal consistency for HADS-D ranges between 0.67 to 0.90 (mean score 0.82) (Bjelland et al., 2002).

Participants were also classified in four groups by increasing HADS-D score: normal (0–7), mild (8–10), moderate (11–14) and severe (15–21) (Zigmond & Snaith, 1983).

Statistical analyses

In the sample we selected, 268 subjects (2.6%) had missing data for one or more HADS-D items. These were singly imputed using the Expectation-Maximization algorithm. The imputation model included the HADS-D items and age.

First, we used Student's t-test to compare HADS-D scores between insomnia cases and controls. Secondly, we performed a linear regression with HADS-D as the dependent variable and type of sleep symptoms as an eight-category independent variable, adjusting for sex and age. Differences in HADS-D scores symptoms patterns were compared using Bonferroni's adjustment accounting for all the pairwise comparison between the seven groups. We used bootstrapping with 1000 bootstrap samples, as the data were not normally distributed. Separate analyses for males and females were conducted in the same way.

Frequencies of the insomnia symptoms and their combination were examined across the four HADS-D groups of increasing severity using a chi-squared test. We compared HADS-D scores and age between cases, controls and excluded subjects using ANOVA with 1000 bootstrap samples. A chi-squared test was used to examine differences in distribution of sexes among the three groups. All analyses were conducted using IBM SPSS 25 (SPSS Inc., Chicago, IL, USA).

Results

In our sample ($N = 7933$) 66.2% were females. Of these 64% percent reported symptoms of insomnia as compared to 48.2% of males ($\chi(1) = 144$, 6, $p < 0.001$). Mean age for the sample was 49.7 years, (SD = 16.2, range: 19.2 to 96.8). The mean age for cases was 54 years while for controls it was significantly lower by 9 years, ($t(7931) = 26.29$, $p < 0.001$). HADS-D score was significantly higher in cases (M = 4.1, SD = 3.2) than controls (M = 1.9, SD = 2.2), ($t(7931) = 35.66$, $p = 0.001$).

HADS-D scores were significantly higher for males than females in both cases (M = 4.7 vs 3.9) ($t(1945) = -7.25$, $p < 0.001$) and controls (M = 2.2 vs 1.7) ($t(3123.5) = -6.18$, $p < 0.001$). Among cases, HADS-D was higher for males independently from the pattern of symptoms experienced (Table 1).

The regression results showed that the HADS-D scores differed significantly among the types of insomnia symptoms ($F(6, 4317) = 27.35$, $p < 0.001$). Table 2 shows the results for Bonferroni-corrected group comparisons. Participants reporting all three insomnia symptoms had the highest depression score (M = 5.2, SD = 3.6), followed in decreasing order by sleep onset problems with terminal insomnia (M = 5, SD = 3.4), sleep onset insomnia with sleep maintenance insomnia (M = 4.6, SD = 3.2), sleep maintenance insomnia with terminal insomnia (M = 4.3, SD = 3.1), terminal insomnia (M = 4.1, SD = 3), sleep onset insomnia only (M = 4, SD = 3.2), and sleep maintenance insomnia only (M = 3.4, SD = 2.9). Mean HADS-D score was significantly lower for maintenance insomnia than for all other patterns of symptoms. Other groups significantly different from one another are reported in Table 2. Inclusion of sex and age as covariates $F(6, 4317) = 28.7$, $p < 0.001$) did not significantly change the mean differences among the groups as shown in Table 3 by the 95% CI.

The pattern of results was different when conducting separate analyses for men and women. In particular, men experiencing sleep onset and terminal insomnia simultaneously had HADS-D scores that are significantly higher than men reporting any other patterns of symptoms (mean difference of 3 points). In females, the same combination of sleep onset insomnia and terminal insomnia differed significantly only from sleep maintenance insomnia ($p = 0.004$, mean difference = 1.12 95%CI [0.01 to 2.23]) (Table 4).

The distribution of insomnia symptoms according to the four HADS-D levels is shown in Fig. 1. The percentage of people experiencing sleep maintenance problems were significantly higher in the "Normal" HADS-D group (33%) than all the others ($p < 0.001$). Regarding all three symptoms of insomnia, only 13% in the "Normal" group experienced this pattern while much higher proportions in the other groups with a peak of 41% in the "Severe" group.

The 10,540 subjects excluded from the study had a mean HADS-D score of (M = 2.9), which is between controls

Table 1 Descriptive statistics for the sample we investigated in this study

		Sleep onset (SOI)	Maintenance (MI)	Terminal (TI)	SOI + MI	MI + TI	SOI + TI	All symptoms	Tot. Cases	Controls	Total
N		*733*	*1333*	*470*	*516*	*527*	*96*	*642*	*4317*	*3616*	*7933*
Females %		*72*	*71.6*	*62.1*	*80.8*	*69.6*	*80.2*	*81.9*	*73.2**	*57.9*	*66.2*
Age M (SD)		*49.5 (17.8)*	*53.3 (16.3)*	*57.9 (15.2)*	*52.6 (15.3)*	*56.2 (14.2)*	*59.7 (18.1)*	*56.1 (15.4)*	*53.9 (16.2)**	*44.6 (14.7)*	*49.7 (16.2)*
HADS-D M (SD)	**Tot.**	*4.0 (3.2)*	*3.4 (2.9)*	*4.1 (3.0)*	*4.6 (3.2)*	*4.3 (3.1)*	*5 (3.4)*	*5.2 (3.6)*	*4.1 (3.2)**	*1.9 (2.1)*	*3.2 (2.8)*
	Females	*3.7*[a]	*3.2*[a]	*3.9*[a]	*4.4*[a]	*4.0*[a]	*4.3*[a]	*5.0*[a]	*3.9*[a]	*1.7*[a]	*2.8*[a]
	Males	*4.5*	*4.1*	*4.6*	*5.3*	*5.0*	*8.1*	*6.2*	*4.7*	*2.2*	*3.3*

*significantly different (α = 0.05) between cases and controls
[a]significantly lower (α = 0.05) than in males

(M = 1.9) and cases (M = 4.2). Age followed a different trend, with excluded participants presenting the highest mean age (M = 54), followed by cases (M = 52) and controls (M = 45). Finally, excluded subjects showed a percentage of females than is higher (73.2%), than the others groups (66.3% in cases and 58% in controls).

Discussion

In this study, we analysed differences in levels of depressive signs among individuals reporting different patterns of nocturnal symptoms of insomnia. We found HADS-D scores to be lower in controls compared to insomnia cases and there were several statistically significant differences between patterns of symptoms. These differences were greater for men than women.

Individuals reporting symptoms of insomnia scored an average of 2.2 points higher than controls. This difference was statistically significant and can be considered meaningful as it is over the minimal important difference (MID) for HADS-D, estimated to be between 1.9 to 2.3 points (Chan et al., 2016). Moreover, these results are in line with the notion that insomnia is associated

Table 2 Pairwise comparison of mean HADS-D for each combination of symptoms

Pairwise comparison		Mean difference	95% CI	*p*-value *
Onset	**Maintenance**	0.5	[0.1 to 1]	**0.009**
	Terminal	− 0.2	[− 0.8 to 0.4]	1.000
	Onset + Maintenance	−0.6	[−1.2 to − 0.1]	**0.007**
	Maintenance + Terminal	− 0.3	[− 0.9 to 0.2]	1.000
	Onset + Terminal	− 1.1	[− 2.1 to − 0.1]	**0.030**
	Onset + Maintenance + Terminal	− 1.3	[− 1.8 to − 0.8]	**< 0.001**
Maintenance	**Terminal**	− 0.7	[− 1.2 to − 0.2]	**0.001**
	Onset + Maintenance	− 1.2	[− 1.7 to − 0.7]	**< 0.001**
	Maintenance + Terminal	− 0.9	[− 1.4 to − 0.4]	**< 0.001**
	Onset + Terminal	− 1.6	[− 2.6 to − 0.6]	**< 0.001**
	Onset + Maintenance + Terminal	− 1.8	[− 2.2 to − 1.3]	**< 0.001**
Terminal	**Onset + Maintenance**	− 0.5	[− 1.1 to 0.2]	0.469
	Maintenance + Terminal	− 0.2	[− 0.8 to 0.4]	1.000
	Onset + Terminal	− 0.9	[− 2 to 0.2]	0.228
	Onset + Maintenance + Terminal	− 1.1	[− 1.7 to − 0.5]	**< 0.001**
Onset + Maintenance	**Maintenance + Terminal**	0.3	[− 0.3 to 0.9]	1.000
	Onset + Terminal	−0.4	[− 1.5 to 0.6]	1.000
	Onset + Maintenance + Terminal	− 0.6	[− 1.2 to − 0.1]	**0.018**
Maintenance + Terminal	**Onset + Terminal**	−0.7	[− 1.8 to 0.3]	0.718
	Onset + Maintenance + Terminal	− 0.9	[− 1.5 to − 0.4]	**< 0.001**
Onset + Terminal	**Onset + Maintenance + Terminal**	−0.2	[− 1.2 to 0.9]	1.000

*Bonferroni corrected for 21 comparisons
N.B.: Differences in mean values can be considered as effect measure for the pairwise comparisons (Baguley, 2009)

Table 3 Pairwise comparison for mean HADS-D scores among pattern of symptoms with sex and age as covariates

Pairwise comparisons		Difference in HADS-D mean	95% CI	*p*-value *
Onset	**Maintenance**	0.7	[0.2 to 1.1]	**0.001**
	Terminal	0.2	[−0.4 to 0.8]	1.000
	Onset + Maintenance	−0.6	[− 1.3 to 0.0]	0.108
	Maintenance + Terminal	−0.1	[− 0.8 to 0.5]	1.000
	Onset + Terminal	− 1.7	[−3 to − 0.5]	**0.001**
	Onset + Maintenance + Terminal	− 1.3	[− 1.9 to − 0.7]	**< 0.001**
Maintenance	**Terminal**	− 0.5	[− 1.0 to 0.0]	0.100
	Onset + Maintenance	− 1.3	[− 1.9 to − 0.7]	**< 0.001**
	Maintenance + Terminal	− 0.8	[− 1.3 to − 0.3]	**< 0.001**
	Onset + Terminal	−2.4	[− 3.6 to − 1.1]	**< 0.001**
	Onset + Maintenance + Terminal	− 1.9	[− 2.5 to − 1.4]	**< 0.001**
Terminal	**Onset + Maintenance**	− 0.8	[− 1.5 to − 0.9]	**0.015**
	Maintenance + Terminal	− 0.3	[− 0.9 to 0.3]	1.000
	Onset + Terminal	− 1.9	[− 3.2 to − 0.6]	**< 0.001**
	Onset + Maintenance + Terminal	− 1.4	[− 2.1 to − 0.8]	**< 0.001**
Onset + Maintenance	**Maintenance + Terminal**	0.5	[− 0.2 to 1.2]	0.731
	Onset + Terminal	− 1.1	[− 2.4 to 0.2]	0.191
	Onset + Maintenance + Terminal	− 0.6	[− 1.4 to 0.1]	0.112
Maintenance + Terminal	**Onset + Terminal**	− 1.6	[− 2.9 to − 0.3]	**0.003**
	Onset + Maintenance + Terminal	− 1.1	[− 1.8 to − 0.5]	**< 0.001**
Onset + Terminal	**Onset + Maintenance + Terminal**	0.5	[− 0.8 to 1.8]	1.000

*Bonferroni corrected for 21 comparisons
N.B.: Differences in mean values can be considered as effect measure for the pairwise comparison (Baguley, 2009)

with depression as reported by, among others, a meta-analysis from 2016 (Li et al., 2016).

HADS-D results were statistically different also among several patterns of symptoms. Experiencing terminal insomnia alone (M = 4.1), did not differ in HADS-D score significantly from experiencing only sleep onset problems (M = 4.0). However experiencing these two symptoms at the same time produced significantly higher average HADS-D score (M = 5.0). As previously suggested (Bragantini et al., 2019), this combination of symptoms might affect sleep length more than other patterns and therefore produce more severe consequences (Vgontzas et al., 2013). In a previous article, we reported a similar results also for the anxiety subscale of HADS (HADS-A) (Bragantini et al., 2019). This suggests that experiencing sleep onset problems together with terminal insomnia reflects a more severe psychological distress than for other patterns of symptoms.

This combination of symptom may be the result of a peculiar etiopathology or of the presence of different, concomitant factors. Previous studies reported sleep onset insomnia increasing the risk for debilitating mental problems (Canivet et al., 2014) while neuroimaging brings evidence of a connection between early morning awakenings and depression (Stoffers et al., 2012; Yu et al., 2018). Speculatively, it is possible that the two insomnia symptoms and depression are connected in a consecutive and escalating manner. Early morning awakening may be a symptom of depression from the beginning due to functional changes in the OFC while sleep onset could emerge when depression exacerbate.

Surprisingly, experiencing also the third symptom, maintenance problems, did not significantly increase the HADS-D score (M = 5.2). This is consistent with the fact that maintenance insomnia has the lowest HADS-D score (3.4 points), which was the lowest score among the patterns of symptoms. However, the difference among maintenance insomnia and other single symptoms was statistically significant but not meaningful, as it was only 0.5 point with sleep onset insomnia and 0.7 point with terminal insomnia. This is in agreement with previous studies in which single nocturnal symptoms of insomnia did not differ in measure of depression (Taylor et al., 2005; Pillai et al., 2015).

Analysing the data in another perspective gave a similar view. When participants were divided into four groups according to increasing HADS-D scores, among people in the "Normal" group, 33% reported maintenance insomnia. This percentage was significantly higher than for all other groups. On the other hand, the

Table 4 Post-hoc analyses for differences among pattern of symptoms in HADS-D scores stratified by gender

Pairwise comparisons		Women			Men		
		Mean Difference	95% CI	*p*-value	Mean Difference	95% CI	*p*-value
Onset	**Maintenance**	0.571*	[0.06 to 1.08]	0.01	0.37	[−0.49 to 1.23]	1.00
	Terminal	−0.157	[−0.84 to 0.52]	1.00	−0.05	[− 1.07 to 0.97]	1.00
	Onset + Maintenance	−0.693*	[− 1.3 to − 0.08]	0.01	− 0.82	[− 2.04 to 0.39]	0.84
	Maintenance + Terminal	−0.27	[− 0.9 to 0.36]	1.00	− 0.47	[− 1.52 to 0.58]	1.00
	Onset + Terminal	− 0.55	[− 1.69 to 0.59]	1.00	− 3.60*a	[− 5.99 to − 1.22]	0.00
	Onset + Maintenance + Terminal	−1.28*	[− 1.86 to − 0.71]	0.00	− 1.65*	[− 2.81 to − 0.5]	0.00
Maintenance	**Terminal**	− 0.73*	[− 1.35 to − 0.1]	0.01	−0.42	[−1.32 to 0.49]	1.00
	Onset + Maintenance	−1.26*	[−1.81 to −0.72]	0.00	− 1.19*	[− 2.31 to − 0.07]	0.03
	Maintenance + Terminal	− 0.84*	[−1.41 to − 0.27]	0.00	−0.84	[− 1.78 to 0.1]	0.13
	Onset + Terminal	− 1.12*	[− 2.23 to − 0.01]	0.04	− 3.97*	[− 6.31 to − 1.63]	0.00
	Onset + Maintenance + Terminal	−1.85*	[− 2.36 to − 1.35]	0.00	− 2.02*	[− 3.08 to − 0.97]	0.00
Terminal	**Onset + Maintenance**	− 0.54	[−1.25 to 0.18]	0.46	− 0.77	[− 2.02 to 0.47]	1.00
	Maintenance + Terminal	− 0.11	[− 0.85 to 0.62]	1.00	− 0.42	[− 1.51 to 0.66]	1.00
	Onset + Terminal	− 0.39	[− 1.59 to 0.8]	1.00	−3.55*a	[−5.95 to − 1.16]	0.00
	Onset + Maintenance + Terminal	−1.13*	[− 1.81 to − 0.44]	0.00	−1.60*	[− 2.79 to − 0.42]	0.00
Onset + Maintenance	**Maintenance + Terminal**	0.42	[−0.25 to 1.09]	1.00	0.35	[−0.92 to 1.62]	1.00
	Onset + Terminal	0.14	[−1.01 to 1.3]	1.00	−2.78*a	[− 5.27 to −0.29]	0.01
	Onset + Maintenance + Terminal	−0.59	[− 1.2 to 0.02]	0.07	− 0.83	[− 2.19 to 0.53]	1.00
Maintenance + Terminal	**Onset + Terminal**	− 0.28	[− 1.45 to 0.89]	1.00	− 3.13*a	[− 5.54 to − 0.72]	0.00
	Onset + Maintenance + Terminal	−1.01*	[− 1.65 to − 0.38]	0.00	−1.18	[− 2.39 to 0.03]	0.07
Onset + Terminal	**Onset + Maintenance + Terminal**	− 0.73	[−1.87 to 0.41]	1.00	1.95	[−0.51 to 4.41]	0.34

N.B.: Differences in mean values can be considered as effect measure for the pairwise comparisons (Baguley, 2009)
*p-value <0.05
[a]statistically different (p<0.05)

percentage of people reporting all three symptoms of insomnia was significantly lower in the HADS-D "Normal" group (13%).

In general, maintenance insomnia seems to be a more common complaint than other symptoms (Canivet et al., 2014; Bragantini et al., 2019; Taylor et al., 2005) but is associated to lower psychological distress in this and our previous study on anxiety (Bragantini et al., 2019). Accordingly, artificially produced sleep fragmentation seems to have only a limited effect on increasing cortisol levels both at night and the morning after (Späth-Schwalbe et al., 1991; Hucklebridge et al., 2000). After an initial burst of cortisol at the introduction of sleep disruption, the individuals seems to habituate and cortisol levels decrease at following interruptions. These differences could be related to the phase in which sleep is disrupted (i.e. N-REM or REM) or to accumulation of sleep time counterbalancing the stress of the interruptions (Späth-Schwalbe et al., 1991).

Alternatively, this symptom may be the manifestation of other organic sleep disorders rather than correlate of psychological distress. Conditions such as sleep apnoea (SA), restless leg syndrome (RLS) and other disorders may disrupt sleep several times during the night (Bonnet & Arand, 2003) without leading to severe depression or anxiety.

Previous studies found that individuals reporting "combined insomnia" (i.e. sleep onset and maintenance insomnia) scored significantly higher than those with single symptoms in measures of depression (Taylor et al., 2005; Pillai et al., 2015). Similarly, in our study, participants reporting combined sleep onset and maintenance/terminal insomnia had higher scores, which was statistically significant, than respondents with only one

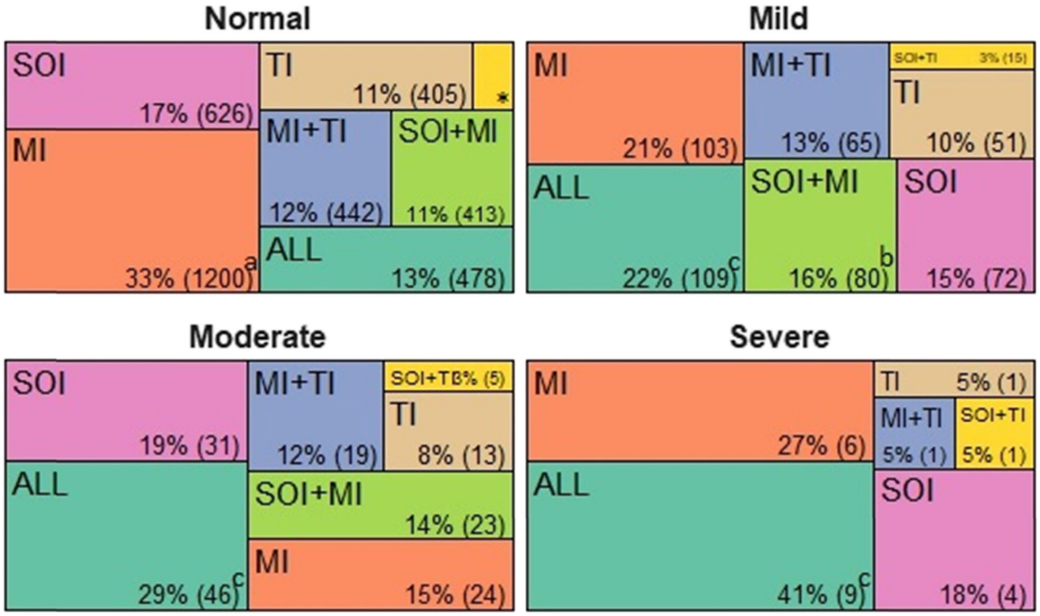

Fig. 1 Comparison of the distribution of patterns of symptoms of insomnia among the HADS-D subgroups. [a] = $p < 0.05$ for comparison of percentages within all other HADS-D groups. [b] = $p < 0.05$ for comparison of percentage of individuals experiencing Onset + Maintenance in HADS-D group "Normal". [c] = $p < 0.05$ for comparison of percentage of individuals experiencing Onset + Maintenance + Terminal in HADS-D group "Normal". **SOI + TI*, 2%

symptom, except for terminal insomnia. This may indicate that the burden of sleep onset insomnia is somehow magnified only in the presence of another symptom. As discussed earlier, maintenance insomnia may be the result of somatic diseases. The relatively low psychological burden of these conditions could interact negatively with the one provoked by concomitant sleep onset insomnia, intensifying symptoms of depression.

In contrast to previous findings, in our sample, men had more pronounced HADS-D scores (M = 3.3). A meta-analysis from 2017 showed that women are two times more likely than men to be diagnosed with Major Depressive Disorder (MDD) and 1.6 times to have depressive symptoms (Salk et al., 2017). Even so, the inverted trend of HADS-D scores have been described before and appear to be a peculiarity of the HUNT cohort. Reasons for this are not clear, but the lack of questions about somatic symptoms of depression in HADS-D, which are more frequent in women, are hypothesized to be the cause for this unusual trend (Langvik et al., 2016).

The lack of somatic symptoms of depression in HADS-D questionnaire results in an over-representation of the anhedonic symptoms. These are typical of the melancholic depression sub-type (Fletcher et al., 2015), characterized also by sleep difficulties, specifically in the form of early morning awakenings (American Psychiatric Association, 2013). From our results we cannot confirm this notion which seems to emerge from clinical practice more than scientific literature. Future studies could deepen the knowledge on the relationship between insomnia and depression by including also measures for single symptoms of depression. This approach could bring clarity over which symptoms of depression and which symptoms of insomnia are more tightly correlated.

Strengths, limitations and future prospective

In this study, we were able to analyse all possible patterns of nocturnal symptoms of insomnia thanks to the use of data from the HUNT study. Moreover, free healthcare and strong welfare measures in the Norway reduced the confounder potential of socioeconomic factors.

The HUNT3 study sleep questionnaire presented only the three aforementioned questions on nocturnal symptoms of insomnia. Sleep length, sleep satisfaction and duration of the sleep problems, were not present in the HUNT dataset and therefore we could not produce a more defined definition of the three nocturnal symptoms of insomnia.

Even if we did not find large differences in HADS-D among people experiencing different patterns of

symptoms of insomnia, the picture may be different in a clinical sample. In future studies, focusing on patients diagnosed with insomnia using tools with higher resolution may highlight these differences.

The approach of stratifying results of studies on insomnia by nocturnal symptoms could improve the knowledge on insomnia on several levels. In particular, identifying the specific psychopathological characteristics of the symptoms and integrating them with findings from sleep research could help elucidate the neurobiological mechanisms that link insomnia and depression. Moreover, identifying which symptoms of insomnia are more likely to be accompanied by severe depressive symptoms may be a useful information in the clinic. For example, patients reporting the nocturnal symptoms associated with more depressive symptoms could be monitored more closely for depression. At the same time, the therapeutic offer could also be tailored to the reported symptoms of insomnia to include interventions that target depression.

Conclusions

Different patterns of nocturnal symptoms of insomnia are associated with different levels of depression. Of relevance, people with sleep onset insomnia combined with terminal insomnia had the highest depression levels, but reporting maintenance insomnia in addition did not increase the HADS-D scores any further. Overall, individuals experiencing maintenance insomnia alone had the lowest scores on the HADS-D. This suggests that the relationship between depression and insomnia may vary according to the pattern of symptoms experienced. Further studies in clinical samples may help reveal relevant differences among patterns of symptoms, which may aid in refining interventions for concomitant depression and insomnia.

Abbreviations

DSM-5: Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition; FDR: False Discovery Rate; HADS: Hospital Anxiety and Depression Scale; HUNT: Helse Undersøkelse Nørd-Trøndelag (Nord-Trøndelag Health Study); MDD: Major Depressive Disorder; MID: Minimal important difference; MRI: Magnetic Resonance Imaging

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Authors' contributions

DB: analysis of the data and draft of the manuscript; BS: supervision of the study; PG: critical reading and discussion of manuscript; SL: design and interpretation of the statistical methodology and results; ICG: design and supervision of the study; All authors: critical revision of the manuscript and contribution to the intellectual content of the work. The authors read and approved the final manuscript.

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Availability of data and materials

The data used in this study are available on request to the HUNT databank.

Ethics approval and consent to participate

This study was approved by the Regional Committee for Medical and Health Research Ethics of South-East Norway (reference number 2016/672) on date 04.27.2016. All partakers in the HUNT study signed a written informed consent form allowing the use of their data and samples for research purposes. Participants can request to withdraw their data from the HUNT database at any given moment.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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