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Pharmacogenetics of morphine in cancer pain

Thesis for the degree of Philosophiae Doctor

Trondheim, May 2009

Norwegian University of Science and Technology
Faculty of Medicine
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ISBN 978-82-471-1668-5 (printed ver.)
ISBN 978-82-471-1669-2 (electronic ver.)
ISSN 1503-8181

Doctoral theses at NTNU, 2009:140

Printed by NTNU-trykk

Farmakogenetikk og morfin i smertebehandling for kreftpasienter

I den industrialiserte delen av verden øker antall personer som får kreft årlig, hovedsakelig på grunn av økt levealder. En av tre nordmenn vil i løpet av sin levetid utvikle kreft. Smertebehandling kan være nødvendig ved alle stadier av en kreftsykdom, og er viktig for å sikre pasienten best mulig livskvalitet. Morfin anbefales som førstevalg av opioider for behandling av moderate til sterke kreftsmerter. Imidlertid vil 10 -30 % av pasientene ikke oppnå tilfredsstillende effekt av morfin, enten på grunn av uholdbare bivirkninger eller fortsatt smerte – eller en kombinasjon av begge. I de tilfeller hvor morfin virker godt, er det dessuten stor variasjon i dosen morfin som trengs for å oppnå analgetisk effekt. En årsak til slik interindividuell variasjon kan være variasjon i genene. Hvor mange gener som er involvert, og hvilke variasjoner det dreier seg om, er ikke klarlagt.

Ved farmakogenetiske studier finner man ut hvordan variasjon i gener kan påvirke effekt og eventuelle bivirkninger av medikamenter. I denne doktorgraden har vi forsøkt å finne ut om det er noen gener som påvirker morfinresponsen i en populasjon av kreftpasienter med smerter. Vi har sett på to systemer: μ-opioid reseptor systemet og catechol-O-metyltransferase systemet. Morfin og andre klinisk viktige opioider bindes primært til μ-opioid reseptoren. Derfor er genet som koder for μ-opioid reseptoren (*OPRM1* genet) viktig. Catecholaminer er involvert i modelleringen av smerte og er delvis metabolisert av enzymet, catecholamin-O-metyltransferase (COMT). Det er dokumentert at variasjon i *COMT* genet kan påvirke smertesensitivitet. Hypotesen vår er at både *OPRM1* genet og *COMT* genet kan påvirke responsen til morfin hos kreftpasienter med smerte.

Våre funn indikerer at genetisk variasjon i *OPRM1* genet og i *COMT* genet påvirker den analgetiske effekten av morfin hos kreftpasienter med smerte. Våre data indikerer også at det er interaksjoner mellom to genetiske varianter av *OPRM1* og *COMT* genene, men populasjonen som er studert er for liten til å si noe sikkert om effekten disse to variasjonene har på hverandre. Fenotypen ”morfinrespons i behandling av

kreftsmerter" er kompleks, og har uten tvil et sammensatt bidrag fra flere genetiske faktorer. Det er sannsynlig at flere gener påvirker fenotypen, og vi er kun i startfasen av en reise mot å forstå den komplekse biologien som ligger bak individers respons til opioider.

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Biveiledere: Professor Frank Skorpen og Professor Pål Klepstad

Ovennevnte avhandling er funnet verdig til å
forsvares offentlig for graden ph.d. i molekylær medisin.
Disputasen finner sted i MTA, Auditorium, Medisinsk teksnisk forskningssenter,
fredag 21. august 2009, klokken 12:15

"All the big questions are about relationships!"
From the cartoon guide to statistics by Larry Gonick & Woollcott Smith

Summary

The number of cancer patients in the world is increasing mainly because of ageing populations. In developed countries it is estimated that about one in three will get cancer during their lifetime. In addition to anticancer treatment, pain treatment at all stages of the cancer disease is of high priority and an ongoing challenge in clinical practice. Whilst oral morphine has been the first line drug of choice for moderate to severe cancer pain, 10-30 % of patients treated with morphine do not have successful outcomes, either because of intolerable adverse effects or inadequate analgesia - or a combination of both. Another issue of morphine treatment is that in cases where it does prove efficient, the dose needed to relieve pain varies widely between patients. One explanation of the interindividual variation in response to morphine may lie within the genes. How many genes are involved, and which variation within well studied genes and yet unknown genes, however, is still an unraveled puzzle.

Pharmacogenetics is the studies of how genetic variability influences the responses to drugs. In this thesis, using a pharmacogenetic approach, we have investigated the gene-opioid interaction in patients receiving oral morphine for cancer pain. The genetic focus in this thesis has been on two systems, the μ-opioid receptor system and the catechol-*O*-methyltransferase system. The μ-opioid receptor is the major site for activation of most clinically important opioids, including morphine. Therefore, the gene encoding the μ-opioid receptor (the *OPRM1* gene) was selected for investigation. Catecholamines are involved in the modulation of pain and are partly metabolized by the catechol-O-methyltransferase (COMT) enzyme. Variation within the *COMT* gene is believed to influence pain sensitivity and therefore we hypothesized that the *COMT* gene is a contributor that influences the response of morphine in cancer pain treatment.

Our findings indicate that genetic variation in the *OPRM1* and the *COMT* genes are influencing the analgesic effect of morphine for patients experiencing cancer pain. Our data also indicate that two genetic variants of the *OPRM1* and *COMT* genes display joint effects, but larger cohorts are needed to investigate whether these effects are

enhancing the efficacy of morphine. The phenotype “morphine response in cancer pain treatment” is a multiplex phenotype that has a complex genetic basis. Most likely more than two genes influence the phenotype. We are only at the beginning of the journey towards a better understanding of the complex biology of opioid response.

Content

Acknowledgement

This thesis was carried out at the Department of Cancer Research and Molecular Medicine, Faculty of Medicine, Norwegian University of Science and Technology (NTNU). The primary financial support for this thesis was a grant from NTNU.

Professor Stein Kaasa has been the main supervisor for the project and I would like to thank him for sharing his wisdom, courage and honesty with me. Stein has been an important motivator for me during the PhD graduate period. Professor Frank Skorpen and Pål Klepstad have been my co-supervisors; I would like to thank Frank for his kindness and scientifically support, and Pål for efficient and to the point supervision in the writing of papers.

I want to express my gratitude to all colleagues in the Pain and Palliation Research Group. Special thanks go to Gunn-Heidi Tobekk for always being ready to help, and for organizing to the best for everybody. To my colleagues Kristin Moksnes and Cecilie Baar I am grateful for their friendship and also for sharing ideas and knowledge of scientific value as well as of the meaning of life. I have appreciated inspiring discussions, joyful lunches and late night meetings, and there are a handful of colleagues to thank: Trine Andreassen, Kari Sand, Gunnhild Jacobsen, Ingunn Johansen, Cinzia Marini, Line Oldervoll, Anne Kari Knutsen, Sonja Andersen, Anne Heidi Gjerde and Torill Fladvad.

Paper IV was carried out with financial, technical and scientific support from the Clinical Genomics Group at the Imperial College in London. Especially, I wish to thank Dr Joy Ross, Dr Hiroe Sato and Dr Sophy Gretton for all their help and warmest welcome when I visited in London. I also wish to thank Professor Ken Welsh for giving me the opportunity to collaborate with their brilliant research team.

I am most grateful to Eirik Skogvoll and Peter Fayers for statistical help and discussions regarding Paper II and Paper IV. I am grateful to all my co-authors for successful collaboration.

My dear friends Mari, Siri, Dr. Andreas, Dr. Marianne, and Dr. Mona have given me much attention, understanding, and cheering during my worst and best times as a PhD fellow. We have chosen to stick together like a herd of horses sharing stories, tears and joy in life. I wish to thank my wonderful friends for the wisdom they have shared with me, for a long time, guiding me to choose my way.

I have a team of family members who have contributed to the quality of my thesis. Language vetting of the thesis was done by my intelligent sister Lisa, my professional uncle Olav and my American aunt Jane. My two charming and lovely sons, Ravn and Roald, were born during my time as a PhD student, giving me sleepless nights, baby blues and timetable problems. Nevertheless, having family members pitch in and help with the duties of motherhood has been essential to my completing the PhD. It is with great appreciation that I wish to thank the following persons: my dear mother Turid, my devoted mother-in-law Toril, my aunt Inger and my father-in-law Ivar for patiently taking care of my children during the final hectic months of my work. Finally, without the joyful support and the complete honesty from my dear husband Frode, I would never have started, continued or completed my PhD graduate.

My warmest gratitude goes also to the patients who participated in the studies. Despite their difficult life situations they joined in research projects, thus giving us the opportunity to improve the care for future patients.

Trude Teoline Nausthaug Rakvåg
Trondheim 2009

List of papers

This thesis is based on the following original publications, which are referred to in the text by Roman numerals I-IV.

I. Klepstad, P., Rakvåg, T.T., Kaasa, S., Holthe, M., Dale, O., Borchgrevink, P.C., Baar, C., Vikan, T., Krokan, H.E., Skorpen, F. The 118 A>G polymorphism in the human μ-opioid receptor gene may increase morphine requirements in patients with pain caused by malignant disease. Acta Anaesthesiol Scand 2004; 48: 1232-1239.

II. Rakvåg, T.T., Klepstad, P., Baar, C., Kvam, T-M., Dale, O., Kaasa, S., Krokan, H.E., Skorpen, F. The Val158Met polymorphism of the human catechol-O-methyltransferase (*COMT*) gene may influence morphine requirements in cancer pain patients. Pain 2005; 116: 73-78.

III. Reyes-Gibby, C.C., Shete, S., Rakvåg, T., Bhat, S.V., Skorpen, F., Bruera, E., Kaasa, S., Klepstad, P. Exploring joint effects of genes and the clinical efficacy of morphine for cancer pain: *OPRM1* and *COMT* gene. Pain 2007; 130: 25-30.

IV. Rakvåg, T.T., Ross, J.R., Sato, H., Skorpen, F., Kaasa, S., Klepstad, P. Genetic variation in the catechol-O-methyltransferase (*COMT*) gene and morphine requirements in cancer patients with pain. Molecular Pain 2008; 4: 64.

Abbreviation

ANOVA	Analysis of variance
APS	Average pain sensitivity
BPI	Brief pain inventory
CAS	Coloured analogue scale
CNV	Copy number variation
COMT	Catechol-*O*-Methyltransferase
CYP2D6	Cytochrome P450 2D6
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxy ribonucleic acid
dTTP	Deoxythymine triphosphate
dUTP	Deoxyuridine triphosphate
EDTA	Ethylenediaminetetraacetic acid
EORTC	European Organization for Research and Treatment of Cancer
FRET	Fluorescence resonance energy transfer
GPCR	G-protein coupled receptor
HPS	High pain sensitivity
HRQOL	Health related quality of life
IL-1Ra	Interleukin-1 receptor agonist
Kb	Kilobases
LD	Linkage disequilibrium
LPS	Low pain sensitivity
M3G	Morphine-3-glucuronide
M6G	Morphine-6-glucuronide
Mb	Megabases
MB-COMT	Membrane-bound-COMT
MC1R	Melanocortin-1 receptor
MDR1	Multidrug resistance 1
MMS	Mini mental state
mRNA	Messenger ribonucleic acid
NRS	Numerical rating scale
OPRM1	Opioid receptor, μ variant
PCR	Polymerase chain reaction
S-COMT	Soluble-COMT
SNP	Single nucleotide polymorphism
SPSS	Statistical package for social science
SSP-PCR	Sequence specific polymerase chain reaction
STR	Short tandem repeats
TPMT	Thiopurine-*S*-methyltransferase
UGT2B7	Uridine diphosphate glucuronosyltransferase 2B7
VAS	Visual analogue scale
VRS	Verbal rating scale
WHO	World Health Organisation

1. Introduction

1.1 Translational research

To improve human health, scientific discoveries must be translated into practical applications in the clinic. Such discoveries may begin at “the bench” with basic research where scientists study disease at a molecular or cellular level, then progress to the clinical level - to the patient's “bedside.” Translational research refers to translating research into practice, ensuring that new treatments and research knowledge actually reach the patients for whom they are intended (Woolf 2008). This thesis has been a part of a translation research project with clinicians, genetists, pharmacologists, psychologists, and statisticians working closely together and sharing a main aim of research: Improving the scientific basis for individual pain therapy. A brief introduction to the translational scientific field is given in the chapters below.

1.1.1 Cancer patients and palliative care

Every year about 25,000 Norwegians will be diagnosed with cancer and about a third of the population will get a cancer disease during their lifetime. The four most common forms of cancer are prostate, breast, colon and lung cancer, and the number of new cases has been increasing every year since reporting began in 1953 (Cancer Registry of Norway 2008). These statistics reflect a real increase in the risk of developing cancer disease, but other elements contribute to the increase, such as improved ability to diagnose, and an ageing population. About 80 % of new cancer cases are persons aged over 55 (Cancer Registry of Norway 2008). Growing old is in itself a risk factor for cancer development, due to various risk factors such as environmental influence, gene susceptibility and/or random biological mistakes during DNA replication. In Norway, survival from cancer disease has slightly increased over the last years, with five-year relative survival probabilities of 57% for male and 63% for female cancer patients (Cancer Registry of Norway 2007).

Patients with incurable disease may eventually require palliative care, a medical speciality defined by the World Health Organization in 2002 as:

"Palliative care is an approach that improves the quality of life of patients and their families facing the problems associated with life-threatening illness, through the prevention and relief of suffering by means of early identification and impeccable assessment and treatment of pain and other problems, physical, psychosocial and spiritual" (Sepulveda, Marlin et al. 2002)

However, palliative care is not medicine exclusively for the terminal patient; patients with ongoing anticancer treatment may also benefit from palliative care. Most patients are in need of symptom control also when the cancer disease is the main treatment target (Kaasa 2007).

1.1.2 Pain

Pain, is what the patient says hurts, and defined by the International Association for the Study of Pain (IASP) as:
"An unpleasant sensory and emotional experience associated with actual or defined potential tissue damage, or described in terms of such damage".
For cancer patients, pain is the symptom which is most feared, and affects most aspects of a patient's life, such as physical functioning, daily activity, psychological and emotional status and social life. Eighty percent of patients with advanced cancer disease experience pain, and effective treatment of cancer pain is a high priority and ongoing challenge in clinical practice (McGuire 2004).

Pain can be classified according to its origin, duration or association with a condition. The three main broad categories of pain mechanism are nociceptive, neuropathic and idiopathic. Cancer patients are heterozygous in how pain is experienced and how it appears (Portenoy and Lesage 1999). The existing approaches to pain classification in palliative care are different, mostly not thoroughly validated, and none are widely applied (Knudsen, Aass et al. 2009). The reasons for the lack of systematic and

widespread use of identified classification systems, may be the complexity of pain and the lack of initiatives to reach an international consensus (Kaasa 2007).

1.1.3 Morphine to treat cancer pain

Cancer patients may need pain relief at all stages of the disease. Pain may be the first symptom of a cancer disease and one of the most prevalent symptoms in patients with metastatic disease. The overall aim of pain treatment is to relieve pain to the patient's satisfaction, so that he or she can function effectively and eventually die free of pain (World Health Organization 1996). The three-step-analgesic ladder recommended by the World Health Organization is the current customary practice for management of cancer pain, where opioids remain the drugs of choice for moderate to severe cancer pain (World Health Organization 1996). Oral morphine has been the first-line drug of choice mainly because there is no ceiling effect or upper limit of doses, and it is a naturally occurring pure μ-opioid agonist.

1.1.4 Interindividual variation in morphine responses

The morphine dose needed to relieve pain varies widely between patients (Hanks and Reid 2005). Patients with identical cancer diagnosis, who report pain of equal magnitude, may require considerably different doses of morphine. The reasons for these interindividual differences are many. Psychological factors such as fear of pain or fear of drug effect (Ersek, Kraybill et al. 1999), depression (Laird, Boyd et al. 2008) and cognitive function are some examples of factors that may influence pain sensitivity and thus also influence the wish for morphine. Cancer patients are often treated with several drugs for a variety of symptoms, and drug interactions may contribute to the interindividual variation in morphine dose. Several drugs are reported to have a potential to interact with morphine, mostly suggested to enhance analgesia and give a synergetic effect of morphine (Santillan, Hurle et al. 1998; Wiesenfeld-Hallin 1998; Ross, Wallis et al. 2000), but a drug may also reduce the effect of another drug and bring about a need for higher doses to reach an ideal effect of treatment (Bernard and Bruera 2000).

The fact that genetic variation contributes to variability in drug responses is widely accepted and validated in many research settings (Roden, Altman et al. 2006), so one explanation to the interindividual variation in morphine responses may lie within the genes.

1.1.5 The big step forward in genetic research

From about the 1970s, genetic research was a question of hypothesizing whether selected genes and variation within genes could interact with diseases or other traits, such as drug response. This candidate gene approach led to diagnosis and treatment of patients with rare single gene disorders, such as cystic fibrosis (Kerem, Rommens et al. 1989) and Huntington disease (MacDonald, Ambrose et al. 1993). With the completion of the sequencing of the human genome (Lander, Linton et al. 2001; Venter, Adams et al. 2001), the establishment of genetic variation databases and the concurrent development of powerful techniques, it is now possible to obtain genetic information at rates and volumes never done before. The development of genetic medicine has been said to gradually change from genetics to genomics (Figure 1).

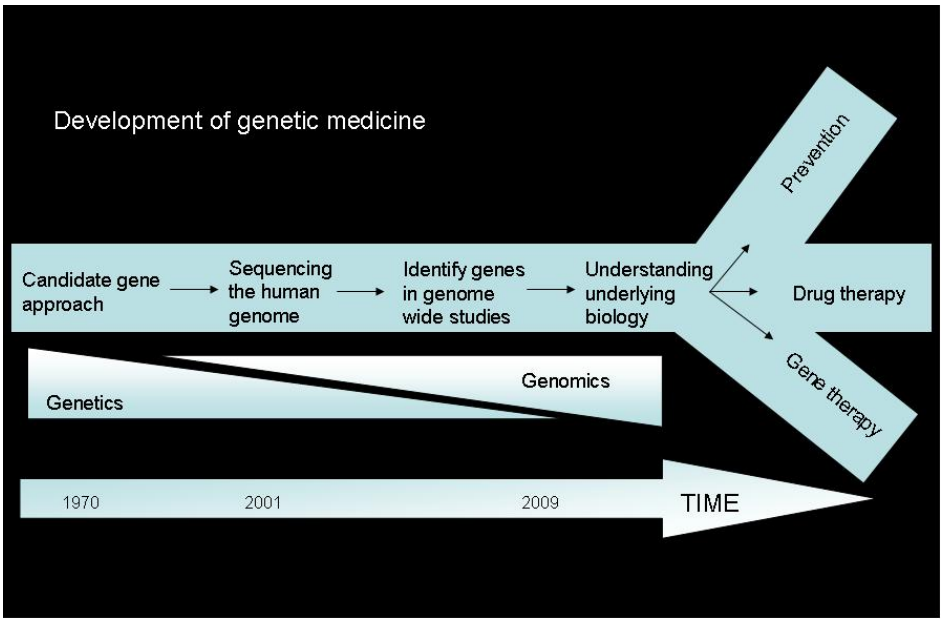

Figure 1: The development of genetic medicine illustrating the shift from genetics to genomics

By definition genetics is the study of single genes and their effects on the body, while genomics is the study of the functions and interactions of all the genes in the genome (Guttmacher and Collins 2002). Genetics will soon (if not already) be out of date, whereas the genomic era has emerged with tremendous speed along with better, faster and cheaper technology. In the genomic era, the focus is to identify genetic contributors to multifactorial diseases and common disorders, that is, diseases resulting from the combined effect of many genes as well as environmental risk factors. These common disorders include, among others, diabetes, cancer, and cardiovascular diseases. The molecular genetic methods for analyses search through the majority of known and validated genes, and variation within these genes. The enormous volume of genetic information obtained with the genomic wide approach has brought about the need for new statistical methods of testing for associations.

This thesis was planned, designed, and conducted at the time when the big step forward in the field of genetic research was taken. The study design was a candidate gene approach and hypothesis testing.

1.2 The pharmacology of opioids

Pharmacology is the study of the interactions that occur between a living organism and exogenous chemicals that alter normal biochemical function. When a drug is given to a patient, it is absorbed and distributed to its site of action, where it will interact with target molecules, undergo metabolism and finally be excreted. Scientists today believe that every pathway of drug metabolism, drug transport and drug effect at receptor level will eventually be found to have a genetic variation (Weinshilboum 2003). However, patient characteristics such as body fat and water stores, age and muscle wasting may also influence the effect of administrated drugs by modifying the amount of opioid in plasma (Paice 2007).

Pharmacokinetics is the study of absorption, distribution, metabolism, and elimination of drugs. The term opioid is defined as a compound, both natural and synthetic, that

have morphine-like actions. The commonly used opioids in pain treatment, including morphine, hydromorphone, oxycodon, fentanyl, and methadone, all differ in their pharmacokinetic properties. They also have quite different chemical structures. Morphine as compared to alternative opioids is cheap, established (well known), safe and has many administrative routes, including oral, subcutanous, rectal, and intravenous. In addition, oral morphine treatment for moderate to severe cancer pain is recommended by the World Health Organization and the European Association for Palliative Care (World Health Organization 1996; Hanks, Conno et al. 2001).

1.2.1 The pharmacokinetics of morphine

Morphine is a naturally occurring alkaloid present in the poppy plant Papaver somniferum, also called the opium poppy. The morphine molecule consists of five condensed rings: phenolic, cyclohexan, cyclohexenol, *N*-methyl-piperidine and a partially saturated furan ring (Figure 2).

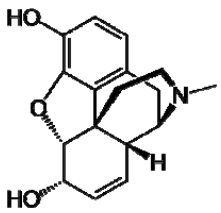

Figure 2: The morphine molecule

Oral morphine is extensively absorbed from the intestines (Milne, Nation et al. 1996). The predominant metabolic fate of morphine is glucuronidation in the liver, where the uridine diphosphate glucuronosyltransferase 2B7 (UGT2B7) enzyme is the catalyst for the biotransformation. The two major metabolites from morphine metabolism are morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G), although there are several other metabolites such as normorphine, normorphine-6-glucuronide, morphine-3-sulfate and morphine-3,6-diglucuronide. However, these substances are found in small concentrations compared to M3G and M6G (Milne, Nation et al. 1996). Of the administrated dose, 55-65% is glucuronidated to M3G, 10-15% to M6G, 5% to other metabolites and 10% is excreted unchanged (Osborne, Joel et al. 1990). M3G and M6G are consistently found in higher serum concentration than morphine during chronic morphine therapy. M6G serum concentration is about six times higher than the serum concentration of morphine and the serum concentration of M3G is even higher

with a factor of about six times the M6G serum concentration (Klepstad, Kaasa et al. 2000). There is large interindividual variability in serum concentration. However, while the M3G/morphine and the M6G/morphine ratios vary widely, the M3G/M6G ratio is more stable (Faura, Collins et al. 1998). Several studies have established that M6G is pharmacologically active and binds to the μ-opioid receptor in the central nervous system producing even more potent analgesic effects than morphine itself (Mercadante 1999), while research has ambiguous answers (Klepstad 2002) to speculations on whether M3G antagonize M6G and morphine induced effects and whether M3G exhibit excitatory effects.

1.2.2 Optimal use of morphine in pain treatment?

The optimal use of morphine in cancer pain treatment is to achieve adequate analgesia without excessive adverse effects. The effective analgesic dose of morphine ranges from as little as 5 mg to more than 1000 mg every four hours, and "finding" the therapeutic window for each patients can therefore be a challenge (Figure 3) (Kaasa 2007).

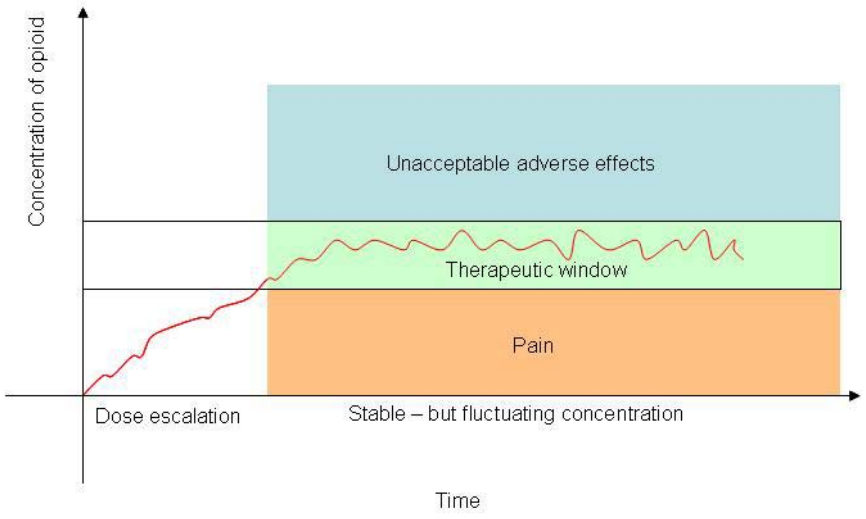

Figure 3: The therapeutic window

The fractions of patients receiving oral morphine that do not reach the therapeutic window range from 10% to 30%. These are patients with intolerable adverse effects, inadequate analgesia, or a combination of both (Cherny, Ripamonti et al. 2001). Adverse effects that prevent further dose escalation and thereby limit the degree of analgesia achieved, are a significant problem in clinical practice, but can often be overcome by switching to an alternative strong opioid.

The different opioids used in pain treatment share an overall mechanism of action by preferentially binding to the μ-opioid receptor. A few genetic terms frequently used in this thesis will be schematically presented before the mechanism of opioid action is described.

1.4 The genetic terms

1.4.1.Glossary

About the gene:

Deoxyribonucleic acid (DNA) is a double helix molecule consisting of 4 bases, adenine (A), thymine (T), guanine (G) and cytocine (C) which together form the molecular basis of genes.

The gene varies widely in length and composition, but within a gene there is always one or more ***exons*** (the coding sequence of DNA), one or more ***introns*** (non-coding sequence of DNA), one or more ***splice sites*** (the junction between an exon and an intron) and one or more ***promoter regions*** (regulatory region of the DNA).

The genome is the collection of all DNA in an organism. The human genome consist of approximately 25 000 genes (Venter, Adams et al. 2001) spread across 23 chromosome pairs.

Locus is sometimes used interchangeably with gene, but refers to a location on the DNA strand.

Allele is defined as the specific variant found on a particular site in the genome. Humans always carry two possible allele variants at a locus, one inherited from the mother and another inherited from the father.

About genetic variation:

A phenotype is a trait, a sometimes visual but always measureable property of an individual, like for instance height, blood pressure, pain, nausea etc. A universal principle in genetics is that virtually all traits have a genetic component.

DNA variations in humans can be microsatellites, short tandem repeats (STRs), single nucleotide polymorphisms (***SNPs***) or insertion or deletion of DNA.

SNPs have been given much attention, and currently 14 708 752 SNPs are registered at the http://www.ncbi.nlm.nih.gov/projects/SNP database, meaning that the human genome on average has 1 SNP every 200 basepair (bp). So far, only about 6 million SNPs are validated, but the field is changing rapidly and more details about SNPs in the human genome are likely to be revealed in the immediate future. SNPs can either have bad, good or indifferent outcomes on the phenotypic level depending on their position at the DNA strand and/or within the gene.

Copy number variation (CNV) is large scale copy number variations in the DNA of 1 kb or larger. CNV can be simple in structure, such as tandem duplications, or may involve complex gains or losses of homologous sequences at multiple sites in the genome (Redon, Ishikawa et al. 2006). CNVs may influence gene dose effects and are therefore suggested to be important contributors to phenotypes.

About classifying genetic groups:

A genotype is defined as the combination of alleles on two chromosomes. Genotype groups will divide into two homozygous groups with two chromosomes of equal DNA information, for example AA and GG, and one heterozygous group of individuals carry one of each allele of the SNP, i.e. AG.

A haplotype is defined as the alleles on different loci carried on the same chromosome, meaning a typical haplotype will be given as a combination of DNA nucleotides, for instance A-T-C-G-G-C-T. The length of the haplotype can either be defined simply by the number of loci analyzed or limited to capture only SNPs that are highly correlated to each other. The latter are said to be within ***haploblocks***.

About two general concepts:

Linkage disequilibrium (LD) is a concept in human genetics that refers to the nonrandom association of alleles at two or more loci (Slatkin 2008). In other words, not all SNPs freely segregate within genes in human populations. D' is the measure of the degree of LD between different loci, and values of D' is on the continuum from -1 to 1. If D'= 1 or -1, there is a complete linkage disequilibrium, and a genotype at one locus can predict the genotype at a second locus. If D'= 0, there is linkage *equilibrium* between loci, meaning that the genotype present at one locus cannot predict the genotype at a second locus.

Epistasis is defined as the interaction between genes (Cordell 2002).

1.5 Pharmacodynamics

Pharmacodynamics, the mechanism of opioid action, is often referred to as "what a drug does to the body". The mechanism of opioid action is to provide analgesia, but also a variety of other physiological effect such as sedation, nausea and vomiting, respiratory depression, confusion, catalepsy, euphoria, and constipation. Opioids act through binding to one or more opioid receptors.

Opioid receptors were demonstrated in the brain by binding studies using radiolabeled opioid ligands in the early 1970s (Pert and Snyder 1973; Simon, Hiller et al. 1973; Terenius 1973). Many receptors were suggested, but as the research field evolved, three of the receptors were regarded as major or "classical" types, μ (mu), κ (kappa) and δ (delta). These receptors were cloned in the early 1990s, and the amino acid sequences of these receptors are about 60% identical to one another. They all share the same general structure of an extracellular N-terminal region, seven transmembrane domains and an intracellular C-terminal tail structure (Satoh and Minami 1995). Most commonly used opioids in pain therapy bind preferentially to the μ-opioid receptor.

1.5.1 The μ-opioid receptor

The μ-opioid receptor is a G-protein coupled receptor (GPCR). Mu (μ) opioids induce analgesia by inhibiting the ascending transmission of nociceptive information and by

stimulating a descending inhibitory system (Basbaum and Fields 1984). More specifically, when an agonist binds to the receptor, the receptor in turn binds to a G-protein and the βγ subunit dissociates from the α G-protein subunit. The free βγ subunit then acts as an effector protein for further signalling and activates a number of second messenger systems, among others: Inhibition of adenyl-cyclase, influx of potassium, and inhibition of calcium channel activity (Connor and Christie 1999). Opioid receptors are expressed both at the spinal level (in the dorsal horn) and in multiple regions within the brain (Stein 1993), but they are also widely expressed in the periphery (Bidlack 2000). Naturally occurring endogenous opioid peptides activate opioid receptors when the body is prepared for a "fight or flight" situation.

Genetic variation in the gene that codes for the μ-opioid receptor (*OPRM1* gene) may influence the pharmacodynamic properties of opioids.

1.5.2 The *OPRM1* gene

The gene encoding the μ-opioid receptor is termed *OPRM1* and is, in humans, located on the long arm of chromosome 6 (6q24-q25). One early model of the murine *Oprm1* gene suggested that it contained one promoter and four exons (Kaufman, Keith et al. 1995). At present, the existence of two promoters (Pan 2002) and 19 exons (Doyle, Sheng et al. 2007), encompassing approximately 250 kb of chromosome 10 is known in mice (Kvam, Baar et al. 2004). Studies on mice have since 1980 indicated the existence of subtypes of the receptor (splice variants), which may be generated by alternative splicing of exons in the μ-opioid receptor gene (Pasternak and Snyder 1975; Pasternak, Childers et al. 1980). The exons (1-19) in mice are known to alternatively spice and generate at least 32 splice variants (Doyle, Sheng et al. 2007). Splice variants of the human μ-opioid receptor have also been identified (Bare, Mansson et al. 1994; Pan, Xu et al. 2005), but at present the functional relevance and expression pattern have mostly been investigated in mice and rats. Future research findings on splice variants of the human μ-opioid receptor will reveal new information about the gene structure, and new exons may be added to the gene model. As the research on splice variants evolves and the complexity of the *OPRM1* gene hopefully will be unravelled, three important

questions can be asked: Do the new exons in the *OPRM1* gene model include genetic variation that influence opioid efficacy? Secondly, does the expression pattern of splice variants of the μ-opioid receptor vary between individuals? And if so, do they possess different binding potential to opioids and do they, as a consequence, influence on opioid efficiency in pain treatment?

1.5.3 Nucleotide variation in the *OPRM1* gene

Hundreds of SNPs have been identified in the μ-opioid receptor gene (dbSNP Build ID: build129, available from http://www.ncbi.nlm.nih.gov/SNP/). Especially, the 118A>G SNP (rs17181017) has been given much attention because it leads to an amino acid shift from asparagine (Asn) to aspartate (Asp) at amino acid position 40 in the protein sequence and is prevalent in most populations. In Caucasians the minor allele frequency is about 16%, but there are considerable ethnic differences; the 118 A>G SNP is significantly less frequent in African-Americans (Hoehe, Kopke et al. 2000). Two other polymorphisms, in intron 2, the IVS2+31 G>A and the IVS2+691 G>C are found in European-Americans with a frequency of more than 10%, where the IVS2+31 G>A polymorphism disrupts one of the (A/T)GGG repeats and therefore may be important for mRNA splicing (Sirand-Pugnet, Durosay et al. 1995). Twenty-four *OPRM1* SNPs have been regarded as candidates for the variability of clinical opioid effects in a review by Lotsch and co-workers, either because they produce an amino acid exchange, are frequent (>1%) or are proposed to have functional consequences indicated in *in vitro* or in human studies (Lotsch and Geisslinger 2005). The review concludes that there are clinical evidence indicating that the 118A>G SNP influence opioid therapy, while the role for other SNPs in the *OPRM1* gene has not yet been shown.

1.5.4 Current literature when the present thesis was planned

One in vitro study suggested a functional effect of the 118 A>G polymorphism, as the G variant of the receptor binds β-endorphin three times more tightly than the A variant (Bond, LaForge et al. 1998). However, these results could not be reproduced by another group (Befort, Filliol et al. 2001). The affinity of all other μ-opioid receptor ligands (including morphine) used experimentally by Bond *et al*., and Befort *et al*., were similar for both receptor variants (Bond, LaForge et al. 1998; Befort, Filliol et al. 2001). In a

study by Lotsch *et al*., 12 healthy volunteers were given intravenous morphine and M6G, and their pupil diameter was assessed as a measure of central opioid effects. Individuals homozygous GG for the 118 A>G polymorphism needed significantly higher doses of M6G to obtain a 50% reduction of pupil diameter compared to homozygous AA individuals (Lotsch, Skarke et al. 2002), but this was not seen for morphine. Sequence variability in the *OPRM1* gene was identified in the promoter, coding regions and in introns (Hoehe, Kopke et al. 2000).

1.6 The catechol-O-methyltransferase (COMT) enzyme

There is evidence to support interaction between dopaminergic and adrenergic pathways and opioid signalling pathways in the central nervous system. Animal studies have shown that a chronic activation of dopaminergic neurotransmission is followed by a reduction in the neuronal content of enkephalin (Steiner and Gerfen 1998), and a compensatory up-regulation of μ-opioid receptors is seen in various regions of the brain (Chen, Aloyo et al. 1993; Steiner and Gerfen 1998).

1.6.1 The physiological function of the COMT enzyme

The catecholamines are neurotransmitters such as dopamine, epinephrine and norepinephrine, which are partly metabolized by the catechol-O-methyltransferase (COMT) enzyme (Axelrod and Tomchick 1958; Guldberg and Marsden 1975). The major physiological function of COMT is to eliminate biologically active or toxic catechols. In addition to its role in the metabolism of catecholamines, COMT is important in the metabolism of several drugs used in the treatment of hypertention, asthma and Parkinson's disease. There exist both a membrane-bound (MB) and a soluble (S) cytosolic form of the COMT enzyme, and at least one of the two distinct transcripts is found in all human tissues examined (Mannisto and Kaakkola 1999). S-COMT dominates in peripheral tissues, such as liver, blood and kidneys, however the ratio of MB-COMT to S-COMT in the brain is about 70:30 (Tenhunen, Salminen et al. 1994; Chen, Lipska et al. 2004). MB-COMT has a higher affinity for dopamine and norepinephrine than S-COMT (Lotta, Vidgren et al. 1995). Together, these results suggest that MB-COMT is well suited to metabolize catecholamines at the concentrations found in the brain (Roth 1992).

Catecholamines have been reported to be involved in the modulation of pain (Ali, Raja et al. 2000; Niemi and Breivik 2002; Pertovaara 2006). Therefore, genetic variability in the *COMT* gene is likely to contribute to differences in pain sensitivity and response to analgesics.

1.6.2 The *COMT* gene

The *COMT* gene is located on the long arm of chromosome 22, at gene map locus 22q11.2. The gene spans approximately 27 kb and contains two promoters, which regulate the synthesis of two distinct transcripts. The most distal 5'promoter (P2) regulates synthesis of a 1.5 kb transcript encoding the membrane associated form of COMT (MB-COMT, 271 amino acids) and the soluble form (S-COMT, 221 amino acids). The second promoter (P1) regulates synthesis of the transcript encoding S-COMT only (Tenhunen, Salminen et al. 1994). The gene contains six exons, of which the first two are non-coding. The MB-COMT and S-COMT are initiated from two separate ATG translation initiation codons in exon 3, but share the same single translation stop codon in exon 6. The coding sequence downstream of the S-ATG start codon is identical for both enzyme forms (Tenhunen, Salminen et al. 1994).

1.6.3 Nucleotide variation in the *COMT* gene

Numerous SNPs have been described in the *COMT* gene, and 22 of the most frequent SNPs distributed throughout the gene have been analysed regarding different aspects of pain and opioid responses (Andersen and Skorpen 2009). The most studied SNP is the rs4680 (also called Val158Met) polymorphism in exon 3 that gives a change from valine (Val) to methionine (Met) at position 108 in S-COMT and at position 158 in MB-COMT. The Met variant of the enzyme is associated with low activity and reduced thermal stability of the COMT protein (Lotta, Vidgren et al. 1995; Lachman, Papolos et al. 1996; Weinshilboum 2006). The low COMT activity appears to be caused primarily by reduced levels of the enzyme, as demonstrated in transfected cells (Shield, Thomae et al. 2004), human liver (Doyle, Goodman et al. 2004; Shield, Thomae et al. 2004) and in brain (Chen, Lipska et al. 2004).

1.6.4 Current literature when the COMT studies were planned

Two studies addressing COMT as a contributor to analgesic effect were performed in this thesis. The first study was based on the knowledge of the rs4680 SNP in COMT and pain modulation. Zubieta and co-workers studied 29 healthy volunteers and exposed them to an intensity-controlled sustained muscular pain challenge. Their hypothesis was that the rs4680 SNP and the low-function Met/Met COMT enzyme would be associated with less capacity to activate μ-opioid neurotransmission under provocative painful conditions by virtue of a lower neuronal content of enkephalin. The authors confirm their hypothesis, and report that individuals with the Met/Met genotype had higher sensory and affective ratings of pain and also higher density of μ-opioid receptors in various brain regions (Zubieta, Heitzeg et al. 2003).

Our second paper addressing COMT as a contributor to analgesic effect was based upon previous studies investigating other SNPs across the *COMT* gene and influences on pain perception (Diatchenko, Slade et al. 2005) and morphine related side-effects (Ross, Riley et al. 2008). Diatchenko and co-workers investigated the relevance of six SNPs along the *COMT* gene, including the rs4680 SNP, and pain sensitivity in 202 healthy female volunteers (Diatchenko, Slade et al. 2005). The authors identified three haplotypes in the *COMT* gene, which they designated low pain sensitivity (LPS), average pain sensitivity (APS) and high pain sensitivity (HPS) haplotypes, based on the their strong association with variation in pain sensitivity. This was measured with experimental pain challenges through pressure, thermal and ischemic pain (Diatchenko, Slade et al. 2005). A study on post-operative pain ratings among 112 European American patients showed associations between maximum post-operative pain ratings and the rs740603 SNP located in intron 1 in the *COMT* gene, but no association was seen for the other 13 SNPs analysed (including the rs4680 SNP) (Kim, Lee et al. 2006). Ross *et al*., found that the rs740603 SNP in intron 1 and a haplotype defined by SNPs in the promoter region and intron 1 were significantly associated with drowsiness and confusion or hallucination in a cancer patient cohort treated with morphine (Ross, Riley et al. 2008). Ross and co-workers did not see any impact of the rs4680 SNP on central side effects, nor did the rs4680 SNP affect morphine requirements among patients.

Taken together, these studies indicate that other SNPs than the rs4680 may be important to investigate, in order to elucidate the effect from COMT on different aspects of pain and analgesic response.

1.7 Pharmacogenetics or pharmacogenomics?

The terms pharmacogenetics and pharmacogenomics tend to be used interchangeably with no precise, consensus definition of either. Nebert *et al*., define the two as follows: Pharmacogenetics is the study of the heritable response to pharmaceutical agents; the study of gene-drug interactions. While pharmacogenomics is the study of how pharmaceutical agents interact with the total expression output of the genome, to influence biological pathways and processes. This latter field should help in designing new drugs (Nebert, Zhang et al. 2008).

Pharmacogenetic studies over the past years have revealed a number of high-penetrance traits of drug response where single genes are recognized as the major explanation for high versus low drug-metabolizing enzymes (reviewed in (Nebert and Vesell 2004)). For some of these monogenetic disorders, genotyping is essential and used in the clinic to prevent known adverse drug reaction. Test for polymorphisms in the thiopurine-S-methyltransferase (*TPMT*) gene is done to avoid severe toxicity to azathiopurine, while the purpose of testing for gene duplication of the cytochrome P4502D6 (*CYP2D6*) is to detect ultra rapid metabolizers or poor metabolizers of debrisoquine (Weinshilboum 2003).

The gene-opioid interaction is multifactorial, as the resulting effect of an opioid is due to the combined effect of many genes, as well as environmental factors as for instance food, other medication, fear of pain or fear of medication, just to name a few. The current evidence for genetic variance influencing the response to opioid in pain treatment is sparse. A limited number of genes have so far been investigated, and studies involve from a few to a couple of hundreds of individuals (Skorpen, Laugsand et al. 2008).

1.7.1 Other genes influencing the efficacy of opioids

The current evidence of genetic variation in genes that may influence the efficacy of opioids in pain treatment has been reviewed in Skorpen and Laugsand (Skorpen, Laugsand et al. 2008). A brief introduction to some of these genes, other than the *OPRM1* and the *COMT* genes, is given below.

The uridine diphosphate-glucuronosyltransferase 2B7 (UGT2B7) enzyme is the predominant catalyzer in the metabolism of morphine to its metabolites, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). A large interindividual variation in the morphine to metabolite plasma ratio has been observed in the clinic. Genetic variation in the *UGT2B7* gene is hypothesized to influence the efficacy of metabolism of morphine. However, the current available evidence suggests that genetic variation in the *UGT2B7* gene does not influence morphine metabolism to a level that is clinically relevant for patients receiving pain treatment (Skorpen, Laugsand et al. 2008).

Interleukins are a group of cytokines (signalling molecules such as hormones and neurotransmitters) that have an important role in inflammatory responses and in pain modulation. One study suggests that genetic variation in the interleukin-1 receptor antagonist (*IL-1Ra*) gene affects the consumption of morphine in post operative pain (Bessler, Shavit et al. 2006).

The melanocortin-1 receptor (*MC1R*) gene is associated with red hair and fair skin phenotype in humans, but the receptor has also been found in neurons of the ventral periaqueductal grey in the brain. These regions are of critical relevance to the modulation of nociception (Xia, Wikberg et al. 1995), and one study reports that genetic variants in the *MC1R* gene affect pain and μ-opioid analgesia in mice and humans (Mogil, Ritchie et al. 2005).

The cytochrom P450 (CYP) consists of a group of monooxygenase isoenzymes located predominantely on the smooth endoplasmatic reticulum membrane in liver hepatocytes

and along the intestinal tract mucosal surface. CYP2D6 accounts for only 2-5% of the total of hepatic P450 isoenzyme, but it catalyzes 25 % of the drugs metabolized, many of which are used in palliative care such as codein and oxycodon (Davis and Homsi 2001). While it is evident that genetic variants in the *CYP2D6* gene influences the efficacy of the weak opioid codein, reports on the influence of oxycodon, a strong opioid increasingly used in palliative care, are sparse and characterized by small studies and case reports (Skorpen, Laugsand et al. 2008).

In order to reach their target of action at opioid receptors, an opioid must cross several membrane barriers, including the blood-brain barrier. There are many drug transporters that theoretically could be involved in the transport of opioids, but the most intensively studied so far is the P-glycoprotein. Interindividual variability in P-glycoprotein activity is well recognized, and genetic variability in the multidrug resistance gene *MDR1*, which encodes the P-glycoprotein, has been reported to be associated with activity differences (Marzolini, Paus et al. 2004). Studies addressing the relevance of genetic variations in the *MDR1* gene are sparse (Skorpen, Laugsand et al. 2008).

2. Aims of the study

The overall aim of this thesis was to investigate the gene–opioid interaction in cancer patients. The thesis focuses on two systems – the μ-opioid receptor (OPRM1) system and the catechol-O-methyltransferase (COMT) system. The following research questions are addressed:

1) Do selected and frequent single polymorphisms (SNPs) in the *OPRM1* gene influence on the opioid requirement in cancer pain patients?
2) Does the frequent polymorphism rs4680 (Val158Met) in the *COMT* gene, which has been associated to the human experience of pain, influence the opioid requirement in cancer pain patients?
3) The 118 A>G SNP in the *OPRM1* gene and the rs4680 (Val158Met) SNP in the *COMT* gene influence the opioid requirement in cancer pain patients. Do gene joint effects of the two SNPs exist?
4) Do haplotypes of the *COMT* gene influence the opioid requirement in cancer pain patients?

3. Material and methods

3.1 Patient cohort

The thesis investigates a cohort originally designed for assessing the relationship between patients' characteristics and serum concentration of morphine and metabolites (Klepstad, Borchgrevink et al. 2003). The study included 300 patients who were treated at the Trondheim University Hospital during the period June 1999 to February 2000. The patients were all diagnosed with malignant disease and received morphine treatment. The study allowed for patients to be included more than once. In the thesis, which investigates genetic variation, we limited our analyses to the first inclusion of each patient, leaving 207 patients available for genetic analyses (Figure 4).

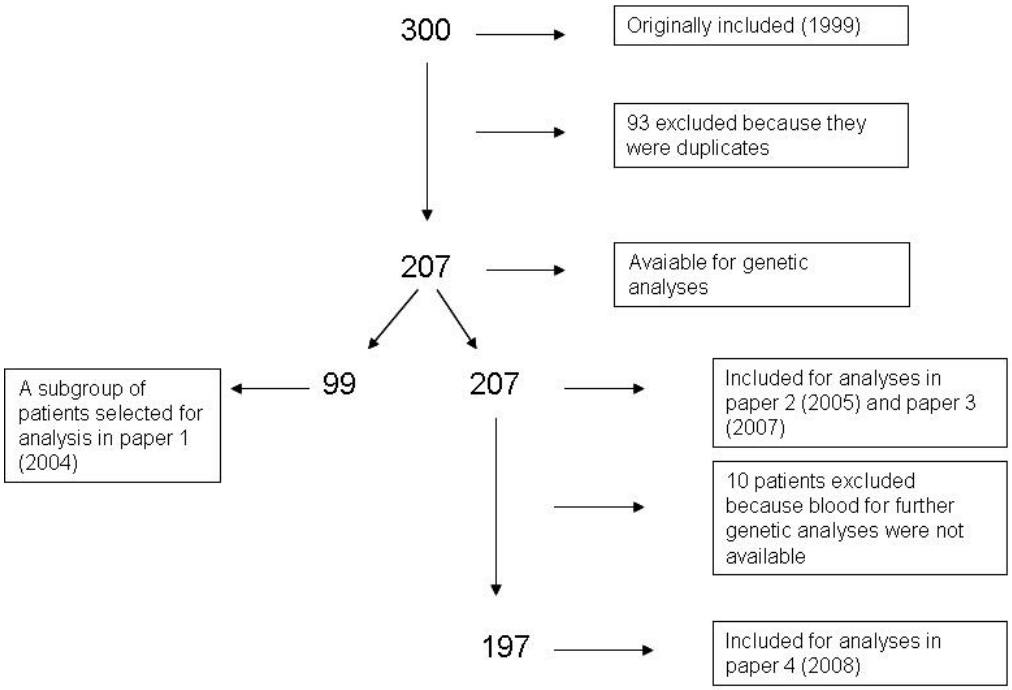

Figure 4: A flowchart of the included patients. Numbers indicate numbers of patients.

A subgroup of 99 patients with good pain control was selected for an analysis investigating the relationship between genetic variants in the μ-opioid receptor gene and efficacy of morphine (Paper I). Patients who reported four or less on the 11-point numeric rate Brief Pain Inventory (BPI) scale item number five, measuring average pain the last 24 hours, were included as this patient subgroup was considered to be adequately treated with morphine.

In the study investigating the relation between a genetic variant of the *COMT* gene and the efficacy of morphine (Paper II), and in the study (Paper III) investigating joint effects of genetic variation found to predict morphine dose for pain control in paper I and paper II, all 207 patients were included. For the final study, where genetic analysis of haplotypes in the *COMT* gene were performed, 197 patients were included because blood for genotyping was no longer available for 10 patients (Paper IV).

Selection criteria for the inclusion of patients are an important issue for any study. The larger the patient cohort is, the stronger statistical power of the analyses. On the other hand, careful considerations of the inclusion criteria of patients are crucial for the validity of the findings. It is of utter importance for genetic association studies that the phenotype investigated is properly defined. For all papers presented in this thesis the validity and comparability of morphine dose requirements between the different genetic subgroups has been of first priority. In spite of the different study designs for the papers in this thesis, we believe that the different genetic subgroups presented are comparable. This assumption is based on the thorough discussion of possible confounding factors in all papers.

3.2 Assessment tools

Assessment tools for measuring pain, patients' health related quality of life (HRQOL), cognitive function, and performance status were assessed for all papers in the thesis. The assessment tools are enclosed in the Appendix. The questionnaires regarding pain and patients' HRQOL were self-reports, whereas cognitive function and performance

status were assessed by the investigators. Methodological considerations of assessment tools used for research purposes should be:

- **Validity:** Does the instrument measure what it intends to measure?
- **Reliability:** Does the instrument produce the same results when repeated on the same population?
- **Inter observer reliability:** Does the instrument produce the same results when repeated by different investigators?
- **Ability to detect changes:** Does the instrument detect clinically meaningful changes?
- **Translation:** Is the instrument formally validated into the appropriate language?
- **Data on the responses in the general population:** Are the responses of the instrument in the general population known?

Symptoms such as pain and questions with psychometric properties are challenging to communicate and measure, and it is difficult to capture the complexity of the biology and psychology in standardized questionnaires. Another important issue in palliative care research is that the assessment tools should be short and easy to complete, because most patients will be significantly physically and mentally reduced during the progress of their disease. The development of a validated assessment tool is time-consuming and exacting and the need for expert opinions and research of existing tools is constant. There are several methods for measuring pain and patients' HRQOL. We have used validated and well recognized assessment tools in this thesis; all of which will be described in the following chapters.

3.2.1 Measuring pain

Pain can be assessed with numeric rating scale (NRS), coloured analogue scale (CAS), verbal rating scale (VRS), or visual analogue scale (VAS). Assessments can either be unidimensional tools targeted to measure one dimension of pain, such as intensity and quality, or multidimensional tools targeted to measure several dimensions of pain, such

as pain intensity in combination with the pain's interference with functions and temporal patterns. In this thesis (all Papers), pain intensity was measured using the item "average pain" during the last 24 hours in the Brief Pain Inventory (BPI) questionnaire. The BPI questionnaire uses a NRS with 11-point alternatives, where 0 represents "no pain" and 10 represents "pain as bad as you can imagine". The BPI is a multidimensional tool and developed for use towards cancer pain patients, validated in Norwegian, and recommended by the European Association of Palliative Care for use in clinical studies (Daut, Cleeland et al. 1983; Caraceni, Cherny et al. 2002; Klepstad, Loge et al. 2002).

3.2.2 Measuring quality of life

The European Organization for Research and Treatment of Cancer core quality-of-life questionnaire (EORTC QLQ-C30) consists of 30 items for patients' self report of function, symptoms and quality of life (Aaronson, Ahmedzai et al. 1993). The psychometric properties and validity of the questionnaire are good (Aaronson, Ahmedzai et al. 1993) and the test/retest reliability is optimal (Hjermstad, Fossa et al. 1995). All scales and single items are linearly transformed giving scores from 0 to 100, where higher score means higher levels of symptoms. Twenty-four items are clustered into multi-item scales: Physical, Role, Cognitive, Emotional, and Social; three symptom scales: Fatigue, Pain, and Nausea and vomiting; and a global health and QOL scale. Finally, there are six single items covering dyspnoea, sleep, appetite, constipation, diarrhoea, and financial impact of the disease and treatment.

Both questionnaires, the BPI and the EORTC QLQ-C30 were delivered and collected directly to and from the patients. This procedure ensured that all questionnaires were returned.

3.2.3 Other assessments

Cognitive function was assessed by the Mini Mental State examination (MMSE). The MMSE scores range from 0 to 30, with higher scores indicating better cognitive function. This is a standardized cognitive screening examination which has proven valid, reliable and able to document changes in cognitive function (Folstein, Folstein et

al. 1975). The feasibility of MMSE has also been demonstrated in studies on patients with terminal cancer (Pereira, Hanson et al. 1997). The patients' functional status was assessed using the Karnofsky performance status score (Karnofsky, Abelmann et al. 1948).

We are aware of the possible confounding effect on the measurement of pain because of patients with low MMSE or Karnofsky scores, and patients who were not able to complete the MMSE. Therefore we reported the Karnofsky and MMSE scores across genotypes in order to reveal any skewed distribution that could lead to a bias of the analyses.

3.3 Pharmacological analyses

All blood samples for the determination of serum concentrations of morphine, M6G and M3G, were obtained during the routine morning round for collection of blood samples. The blood samples were placed in tubes, serum separated by centrifugation, and finally stored at -85°C. Westerling *et al*., have compared the effects of different preanalytical conditions on the measurement of morphine, M6G and M3G serum concentrations, such as different tubes, temperature and time of storage. They found no influence on morphine, M6G or M3G serum concentrations (Westerling, Bengtsson et al. 1996). Thus, the analyses of morphine, M6G and M3G are robust to different handling procedures pre analysis.

In this study, blood samples were not taken at an exact time after morphine administration. This approach was used in order to facilitate the feasibility of clinical routine drug monitoring of morphine. All patients in the presented cohort were on stable doses of morphine, and smaller fluctuation of serum concentration of morphine and metabolites are expected during a dose interval compared to studies for effects after single opioid administration.

3.4 Molecular genetics

Genomic DNA was isolated from 50 to 200 µL EDTA blood on a MagNA Pure LC (Roche Diagnostics Scandinavia AB, Bromma, Sweden) using the MagNA Pure LC

DNA Isolation Kit I and applying the manufacturers high performance protocol. Purified genomic DNA was eluted in 100 µL antiseptic water or elution buffer and stored at – 20 ºC.

3.4.1 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is the gold standard procedure for generating large quantities of a specific DNA sequence *in vitro*, and essential before sequencing or genotyping DNA strands of interest. DNA amplification by PCR, which can be more than a million fold, is achieved by a three-step cycling process including denaturation at 95°C, renaturation at about 60°C and synthesis of DNA at about 70°C. These three successive steps are repeated a number of times, e.g. 40. The essential requirements for the PCR are two synthetic oligonucleotide primers (~ 20 nucleotides each) that can match and pair to specified regions in the target DNA, the DNA sample from an individual, a thermo stable DNA polymerase and the four deoxyribonucleotides, dATP, dGTP, dCTP and dTTP (or dUTP).

3.4.2 Genotyping SNPs

If a SNP is known, frequent and of interest to analyze, genotyping is preferable to DNA sequencing because it is cheaper and less time-consuming. In this thesis, two methods for genotyping were used, a LightCycler PCR system using the fluorescence resonance energy transfer (FRET) method and a sequence specific polymerase chain reaction (SSP-PCR) method.

Genotyping using FRET method

Genotyping with the FRET method was carried out on the LightCycler system. A target DNA which includes the polymorphism of interest is first amplified by PCR. After the PCR run, polymorphism detection is carried out in the same reaction capillary. Two separate 3' and 5' probes are labeled with donor and acceptor fluorophores, and designed to match part of the PCR product, including the polymorphic site. The probes are added in the reaction mix, but will not distract the PCR process.

After the PCR is completed the probes will anneal to its target DNA and an increase in FRET is observed because the donor and acceptor fluorophores get into close proximity (Figure 5).

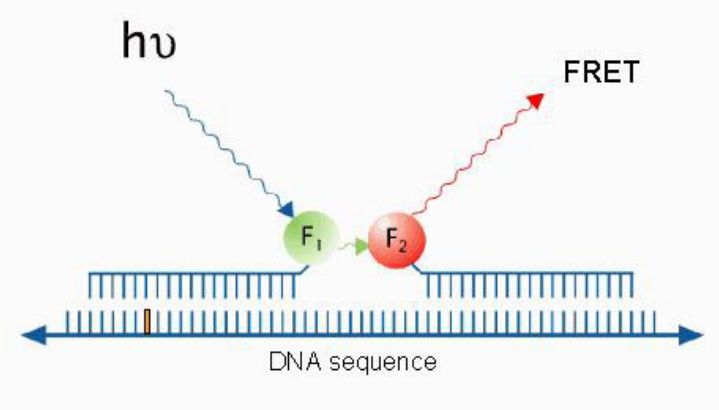

Figure 5: The FRET signal. Two probes, designed for polymorphism detection on the LightCycler, match the DNA sequence in the region where the polymorphism is located. The polymorphism site is indicated by a little orange box in the DNA sequence. The probes are labeled with fluorophores donor (F1) and acceptor (F2). When the probes get in close proximity **hν** (a light source from the LightCycler instrument) will excite F1, which again excites F2 resulting in a FRET signal.

Further on, the reaction tube is heated and the probe/target duplex is denatured. The fluorophores get separated and the FRET signal drops to the background. The sensor probe is designed to have either of the two possible DNA sequences at the polymorphic site. The probe/DNA target duplex will sometimes (depending on the patient's genotype) have a mismatch position which decreases the stability of the duplex. This is reflected by a shift in melting temperature (T_m) visualized on the LightCycler System. As an example, a homozygous AA genotype will generate one T_m peak, a homozygous GG genotype will generate one T_m peak and a heterozygous AG genotype will generate two T_m peaks. This happens because half of the target DNA will match completely with the sensor probe, while the other half will not (Figure 6).

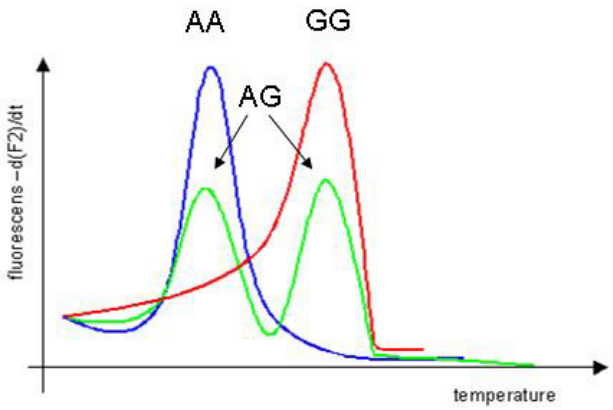

Figure 6: A schematic presentation of an imagined genotyping result from the LightCycler, showing the three possible genotypes (AA, GG and AG) as colored peaks.

Genotyping with the FRET method is reliable, accurate and fast. However, low quality of the DNA, procedure failure by the investigator and some genomic constructions as copy number variation, may cause problems and equivocal results. Genotype results were confirmed with DNA sequencing for each of the SNPs analyzed in the present thesis.

Genotyping using SSP-PCR method

The SSP-PCR method is based on the concept that a sequence specific primer and a consensus primer produce a DNA product of known size in a PCR run. The sequence specific primer has a mismatch at the 3' end, which is designed to identify each genotype variant. Primer concentrations were titrated to ensure amplification only with exact matching of the primer with genomic DNA. Products were visualised with a UV illuminator and photographed with a Polaroid camera. The presence of an allele specific band of the expected size, in conjunction with a control band, was used to identify an allele.

The SSP-PCR method has an overall good resolution and results are easy to interpret. As for genotyping using the FRET method, equivocal genotypes usually relate to the

quality of the DNA, deviations from standard procedures and/or complexity of the human genome.

3.5 Statistical methods

The patient data used in this thesis were originally included into a study designed for assessing the relationship between patients' characteristics and serum concentrations of morphine and metabolites, therefore a prestudy formal sample size calculation was not performed. The Statistical Package for Social Science (SPSS) was used for all analyses in all Papers. The statistical methods that were used vary for the different papers, and detailed descriptions are outlined in each of the presented papers. A brief description is given below.

3.5.1 Statistical analyses for all papers

In Paper I, comparisons between different genotype groups for each of the OPRM1 polymorphisms were performed with one-way ANOVA tests. A p-value less than 0.01 was considered statistical significant in order to give some protection against multiple tests.

In Paper II, comparisons between the different genotypes (Val/Val, Val/Met and Met/Met) were then performed with the Jonckheere-Terpstra test, a non-parametric test working with the hypothesis that $\mu 1 \leq \mu 2 \leq \mu 3$ (or the opposite $\mu 1 \geq \mu 2 \geq \mu 3$). The test was chosen because the *COMT* alleles are expected to be codominant with respect to enzyme activity. In other words, we expected a gene-dose effect of the rs4680 polymorphism in respect to the need for morphine. A p-value less than 0.05 was considered statistical significant.

In Paper III, we performed a multivariable logistic regression analysis using morphine dose as the dependent variable. Odds ratio with accompanying 95% upper and lower bonds are chosen to present results from the logistic regression model of morphine dose.

In Paper IV, we used non-parametric tests for comparisons between genotypes and haplotypes against morphine doses and a stepwise linear regression analysis with the morphine dose as the dependent variable. The Jonckheere-Terpstra test for comparison between genotype groups was used for the rs4680 polymorphism, as explained for Paper II. For all other SNPs we used the Kruskal-Wallis test for comparison between genotype groups. For the analyses of comparisons between haplotypes and morphine doses we used the Mann-Withney U-test. We used the logarithm (log_{10}) of the 24 hour morphine dose as the dependent variable in the regression analyses because the 24 hour morphine dose, as expected, did not display a normal distribution. The analysis was a stepwise enter linear regression with a criterion for removal of a variable of $p>0.1$. The variables included in the regression analysis as independent variables are outlined in Paper IV.

3.5.2 Genotype data

Individual SNPs were examined for genotype and allele frequencies (all papers) and allele carriage (Paper IV). The genotype frequency was calculated by simply counting the number of individuals in the different genotype groups. The allele frequency is the percentage of loci that the allele occupies within the population, and is calculated as shown below.

Allele frequency:
[(2 x #homozygote) + (1 x #heterozygote)] / total #alleles

The allele carriage presents whether an individual carries the allele regardless of being homozygous or heterozygous carrier. The allele carriage is calculated as shown below.

Allele carriage:
#homozygote + #heterozygote / total #individuals

The Hardy-Weinberg principle states that both allele and genotype frequencies in a population remain constant. Consequently, a random genetic sample has a distribution

of homozygous and heterozygous carriers that correspond to the Hardy-Weinberg equilibrium. Violation of the Hardy-Weinberg model may be the result of non-random mating, limited sample size or a possible genotyping system error. The Hardy-Weinberg model compares the observed and expected genotype frequencies, and is based on two equations:

1) $p+q=1$

2) $p^2 + 2pq + q^2 = 1$

Where p is the frequency of one allele; q is the frequency of the other allele; p^2 is the frequency of one homozygous group; 2pq is the frequency of heterozygous individuals; and q^2 the frequency of the other homozygous group.

All genotype frequencies were checked for Hardy-Weinberg equilibrium using the χ^2 -test. Allele frequencies are determined directly from the data available on the population investigated, and the expected genotype frequencies are calculated using the equation number 2 above. Significant ($p<0.05$) differences between expected and observed genotype frequencies indicate that the study population is not in Hardy-Weinberg equilibrium.

3.5.3 Construction of haplotypes (paper IV)

The series of genotype information on a given stretch of DNA is unphased data, which means that we can not tell on which chromosome the series of SNPs lie. Phased data can be established by genotyping family members to infer parental chromosomes, or estimated by the use of different statistical methods. There are two frequently and widely recognized methods for constructing haplotypes; the Expectation-Maximization (E-M) algorithm, and the Bayesian method. In the presented thesis, the haplotypes were predicted using the Bayesian approach with the computer program Phase (http://stephenslab.uchicago.edu/software.html) (Stephens, Smith et al. 2001; Stephens and Donnelly 2003). Phase has shown to offer accuracy of estimation and ability to incorporate genotyping error and/or missing data (Stephens and Donnelly 2003).

3.6 Ethics

The study was carried out in accordance with the principles of the Helsinki declaration. The Regional Committee for Medical Research Ethics, Health Region IV, Norway, approved the study. All patients gave their oral and written informed consent before inclusion in the study. However, a challenge in palliative care research is that a majority of the patients are in a vulnerable situation with advanced cancer disease, and many of them have therefore reduced ability to give informed consent. In palliative research, it is of utter importance to be aware of the difficulties in obtaining adequate informed consent, and observe a special obligation to give patients relevant and understandable information about the study.

The findings of the presented genetic association studies may potentially be of benefit for future patients, because the specified genetic information can be used to improve pain treatment. However, it is possible that not all genetic information is in the patient's best interest, because some genetic variants may predict more than one phenotype. In other words, if genetic tests are future tools in medicine they may carry "good news" about how to facilitate pain treatment, but the same tests may bring "bad news" about possible onset of disease or more likely for instance susceptibility to drug abuse. An ethical discussion pre use of future genetic tests in medicine is therefore essential.

4. Summary of papers

Paper I

The 118 A>G polymorphism in the human μ-opioid receptor gene may increase morphine requirements in patients with pain caused by malignant disease.

Morphine is recommended by the World Health Organization as the treatment of choice for moderate to severe cancer pain. Despite its widespread use, the many side effects and the large individual differences in doses required have complicated its use in clinical practice.

The μ-opioid receptor is the major site for most clinically important opioid drugs, including morphine. The aim of the study was to investigate if selected SNPs in the *μ-opioid receptor* (*OPRM1*) gene influence on the efficacy of morphine in cancer patients with pain. The selection of SNPs was based on the frequency of occurrence in Caucasian population and current literature.

Two hundred and seven patients were genotyped for the well studied 118 A>G SNP, the -172 G>T SNP in the 5'regulatory region, and the IVS2 + 31 A>G and the IVS2 + 691 G>C SNPs in intron 2, all within the μ-opioid receptor gene. A selection of 99 patients, with adequately controlled pain, was compared against morphine doses between genotype groups for all SNPs. We found no differences of morphine doses between genotype groups for the -172 G>T (96±92mg/24h, 108±73mg/24h, 30 mg/24h); the IVS2 +31 (94±87mg/24h, 111±107mg/24h); and the IVS2 + 691 SNPs (99±133mg/24h, 87±68mg/24h, 108±96mg/24h). On the contrary, we found that patients homozygous for the G variant of the 118 A>G SNP needed more morphine (225±143mg/24h) compared to heterozygous individuals and patients homozygous for the A variant of the SNP (97±89mg/24h).

The 118 A>G SNP affects the structure in the extracellular region of the μ-opioid receptor as the substitution from A to G leads to an amino acid shift in the protein from asparagine to aspartate. The 118 A>G SNP has been the most extensively studied

because it is speculated that it may affect the binding efficacy of substrates to the receptor.

Our data indicate that genetic variation in the *OPRM1* gene may influence the efficacy of morphine in a cancer patient cohort. The findings need to be validated in a larger study sample.

Paper II

The Val158Met polymorphism of the human catechol-*O*-methyltransferase (*COMT*) gene may influence morphine requirements in cancer pain patients.

Pain and the efficacy of analgesics are traits of complex genetic basis most likely influenced by a number of genes. The COMT enzyme metabolises the catecholamines: Dopamine, epinephrine and norepinephrine, and is therefore considered a key modulator of dopaminergic and adrenergic neurotransmission. A common and well studied SNP in the *COMT* gene is the rs4680 (Val158Met) polymorphism, because it has been shown to reduce the COMT enzyme activity three-to-four fold. One study, investigating the influence of the rs4680 SNP on pain perception, found that subjects with the Met/Met genotype have the most pronounced response to experimental pain compared to heterozygous Val/Met and Val/Val genotype individuals.

The aim of this study was to find out whether the rs4680 SNP influence on the morphine efficacy for cancer pain. Two hundred and seven patients were genotyped and analyzed for differences in morphine dose requirements, serum concentration of morphine and morphine metabolites. In the presented Norwegian cohort the Met allele, corresponding to the low-activity COMT enzyme, was the most frequent with a relative frequency of 0.56. The morphine dose requirements for the Val/Val, the Val/Met, and the Met/Met genotype groups were 155±160mg/24h, 117± 100mg/24h, and 95±99mg/24h respectively. This difference was statistically significant with p=0.025, and was not explained by other factors such as duration of morphine treatment, performance status, time since diagnosis, perceived pain intensity, adverse symptoms or time until death.

The findings in this study suggest that the rs4680 SNP in the *COMT* gene may contribute to the efficacy of morphine in a cancer patient cohort. This is of principle interest since it demonstrates that genetic variability in genes not directly relevant to opioid systems can influence the efficacy of morphine.

Paper III

Exploring joint effects of genes and the clinical efficacy of morphine for cancer pain: *OPRM1* and *COMT* gene.

Pain and the efficacy of analgesics are traits of complex genetic basis most likely influenced by a number of genes. Two SNPs, the 118 A>G and the rs4680; in two genes, the *OPRM1* gene and *COMT* gene respectively, have been suggested to be modulators of morphine efficacy. The joint effects of genes can be expected to enhance, suppress or have no effect on the phenotypic outcome of interest. The aim of this study was to assess the joint effects of these SNPs in the *OPRM1* and *COMT* genes to the morphine dose requirements in a cancer patient cohort.

We used the genotype data obtained in Paper I and II and clinical data from the original pharmacokinetic study. Two hundred and seven patients were included in a multivariable logistic regression analysis using morphine dose as the dependent variable. In Paper I and Paper II we reported that the A allele (*OPRM1*) and the Met allele (*COMT*) were associated with the lowest morphine dose requirements. In this study we found that carriers of the combination *OPRM1* AA and *COMT* Met/Met genotype, required the lowest morphine doses (87mg/24h; 95%CI=57,116), while patients carrying neither AA nor Met/Met genotype needed the highest morphine doses (147mg/24h; 95%CI=100,108). The differences of morphine dose requirements seen between genotype combinations were significant with a p value of 0.012, even after controlling for demographic and clinical variables in the multivariable analysis.

The findings from this study indicate that there are joint effects from the 118A>G SNP and the rs4680 SNP in the *OPRM1* and the *COMT* gene respectively. Larger cohorts

are needed to investigate whether these effects are enhancing the efficacy of morphine in cancer pain treatment.

Paper IV

Genetic variation in the *Catechol-O-Methyltransferase* (*COMT*) gene and morphine requirements in cancer patients with pain.

Genetic variability in the *COMT* gene is believed to contribute to differences in pain perception and response to analgesics. In Paper II we reported that the rs4680 SNP in the *COMT* gene may influence on the efficacy of morphine in cancer pain treatment. The aim of this study was to investigate if variability in other regions of the *COMT* gene also contributes to the interindividual variability in morphine efficacy. Specifically, we wanted to find out whether haplotypes of the *COMT* gene influence the requirement of morphine in a cancer patient cohort.

Eleven SNPs throughout the *COMT* gene were genotyped, all of which were in Hardy-Weinberg equilibrium. The SNPs were located in the promoter region, in intron 1, 2 and 3, in exon 4, intron 5, and in the untranslated 3' region. They were selected for analyses on the basis of frequency, position within the gene, and what was known from relevant literature. Genotypes and haplotypes were compared with pharmacological, demographical and patient symptoms measurements in 197 cancer patients receiving scheduled oral morphine for cancer pain. Two frequent haplotypes (34.5% and 17.8%) were found in this cohort. Using a multivariate regression method with morphine dose as the independent variable, we found that patients carrying the most frequent haplotype (34.5%) were predicted to need lower morphine doses than patients not carrying this haplotype, with a reduction factor of 0.71 ($p=0.005$). Non-parametric tests show that there were weak associations between allele carriages for six of the SNPs analysed. The six alleles associated with the lowest morphine doses constitute part of the most frequent haplotype.

The findings in this paper suggest that genetic variability in the *COMT* gene influence the efficacy of morphine in cancer patients.

5. Discussion

The overall aim of this thesis was to investigate the gene-opioid interaction for two systems, the μ-opioid receptor and the catechol-O-methyltransferase system. The included papers indicate that genetic variation in both systems may influence on the efficacy of morphine in cancer pain treatment. The findings are a step forward to a better understanding of the complexity of biological factors that contribute to the response of opioids in pain treatment. The following chapters will successively discuss the implication of findings, recent literature, limitations within the papers presented, and general limitation in a pharmacogenetic study design.

5.1 The μ-opioid receptor (Paper I)

Paper I answers the research question number one. Of the four selected SNPs in the *OPRM1* gene, only the 118A>G polymorphism in the *OPRM1* gene influences on morphine requirements in a cancer patient cohort. Patients homozygous for the G allele needed more morphine to achieve pain control compared to heterozygous AG and homozygous AA individuals. The available evidence that suggests a clinical relevance of the G allele of the 118 A>G polymorphism is relatively strong per date. Several studies have addressed the functional consequences of this polymorphism in healthy volunteers.

5.1.1 Studies in healthy volunteers

The study by Lotsch and co-workers, where the 118 A>G polymorphism in the *OPRM1* gene was found to decrease pupil constrictory effect of M6G (Lotsch, Skarke et al. 2002), has been followed up in a study using similar methodology (Skarke, Darimont et al. 2003). In this follow-up study, the effect of the 118A>G SNP on M6G efficacy was confirmed, but they also found that the potency of the pupil-constricting effects of morphine was significantly smaller in subjects carrying the G allele. These individuals also reported less nausea and vomiting after administration of M6G. However, no effect on analgesia was identified in this study, and the importance of the 118 A>G SNP on morphine and M6G efficacy is debated. The authors conclude that pain data are "noisier" than pupil data, and that the study may be too small (12 individuals) to

produce conclusive data (Skarke, Darimont et al. 2003). Two other studies, also addressing the effect of the 118A>G SNP and the efficacy of M6G, showed a reduced analgesic response to M6G in individuals carrying the G allele (Romberg, Olofsen et al. 2004; Romberg, Olofsen et al. 2005), but suggest that the 118 A>G SNP does not protect against opioid induced respiratory depression (Romberg, Olofsen et al. 2005). In sharp contrast to the studies by Romberg *et al*., Oertel and co-workers report that homozygous carriers of the 118 G allele needed 10-12 times higher concentration of alfentanil to elicit the same degree of respiratory depression (Oertel, Schmidt et al. 2006), suggesting that increasing opioid dose in homozygous 118G allele carriers to achieve adequate analgesia is clinically safe. The same study showed that higher alfentanil concentrations (2-4 times) were needed to produce the same degree of analgesia in homozygous carriers of the G allele, compared to homozygous A allele carriers of the 118 A>G SNP (Oertel, Schmidt et al. 2006). Finally, Lotsch and co-workers have reported that pupil constriction, in 51 healthy volunteers receiving levomethadone, are associated to the 118 A>G SNP (Lotsch, Skarke et al. 2006).

To summarize, many studies support each others' findings that there is actually an effect of the 118 A>G SNP on opioid efficacy (Lotsch, Skarke et al. 2002; Skarke, Darimont et al. 2003; Romberg, Olofsen et al. 2004; Romberg, Olofsen et al. 2005; Lotsch, Skarke et al. 2006; Oertel, Schmidt et al. 2006).

5.1.2 Clinical studies

Two recent clinical studies support our findings, by reporting a possible relevance of the *OPRM1* 118A>G polymorphism for post-operative opioid consumption. Both studies report an increased consumption of intravenous morphine (patient-controlled) in patients homozygous GG for the 118 A>G polymorphism, compared to patients homozygous AA (Chou, Wang et al. 2006; Chou, Yang et al. 2006). However, these studies have been criticized because the 118 A>G SNP was not in Hardy-Weinberg equilibrium (Lotsch 2007). Furthermore, the difference reported in morphine consumption (33±10 mg versus 27±9 mg (Chou, Wang et al. 2006)) was questioned by Ruth Landau, who argued that the difference was too small to be of any clinical

relevance, especially since it was only seen the first 24 hours of treatment and disappeared after 48 hours (Landau 2006). Another study, involving 145 cancer patients undergoing pain treatment, indirectly supports our findings (Campa, Gioia et al. 2008). An association between the degree of pain relief and the 118 A>G polymorphism was found; the patients being homozygous carriers of the 118 A allele were associated with the highest pronounced decrease in pain after 7 days of morphine treatment (Campa, Gioia et al. 2008). A recent study investigated the response to morphine for postcesarean analgesia in 588 women, and showed that patients being homozygous carriers of the 118 G allele in the *OPRM1* gene needed the highest doses of morphine to relieve pain (Sia, Lim et al. 2008). The study by Sia *et al*., also clearly supports our findings in Paper I. One study investigating the morphine dose requirements in patients undergoing colorectal surgery, reports no effect of the 118 A>G polymorphism, neither on patient controlled analgesic requirements nor on postoperative nausea or vomiting reports (Coulbault, Beaussier et al. 2006). Finally, one study shows the complete opposite of the above mentioned studies, reporting that women with the 118 G allele may be more responsive to opioids and require less analgesic drugs (Landau, Kern et al. 2008). The design of this study involved intrathecal fentanyl for laboring women and the authors discuss their opposite findings with respect to the effect of the different nature of nociceptive stimulus in labor versus that perceived in experimental models, or in acute postoperative or chronic settings.

Taken together, the available evidence suggests that the 118 A>G SNP in the *OPRM1* gene is associated with reduced opioid effect (as seen for pupil construction, response to experimental pain and respiratory depression) and an increased opioid dosage requirement in patients. However, there seem to be different effects for the different opioid drugs and responses. This is probably due to the complex and numerous biological pathways and contribution of environmental factors and other genes affecting opioid responses.

5.1.3 Functional studies addressing the importance of the 118 A>G SNP

Two likely explanations for the reported effect of the 118 A>G SNP is postulated, both hinting that it might affect the binding potential of the receptor. Firstly, the SNP is located in the gene in such a way that it affects the extracellular region of the receptor were opioids bind. Secondly, the SNP causes an amino acid substitution from asparagine (Asp) to asparatate (Asn), consequently deleting a putative N-glucosylation site and possibly affecting binding efficacy of different molecules. The important question is: Does it influence on opioid binding? The widely cited *in vitro* study by Bond *et al*., showing that β-endorphin had an increased binding affinity with the Asn receptor type (Bond, LaForge et al. 1998), has not been replicated by others (Befort, Filliol et al. 2001; Beyer, Koch et al. 2004; Kroslak, Laforge et al. 2007). Thus, attempts to identify the molecular mechanisms of the clinically observed effects of the 118 A>G SNP on analgesic response have been inconsistent. It has been suggested that the inconsistence in findings may be attributed to the artificial conditions in non-human non-neuronal cells, and also partly to the neglect of region dependent effects of μ opioidergic mechanisms in the human brain (Oertel, Kettner et al. 2008). The presence of the 118 A>G SNP has been reported to decrease analgesic effects of alfentanil at brain regions processing the sensory dimension of pain, but not at brain regions processing the affective dimension of pain (Oertel, Preibisch et al. 2008). Oertel and co-workers investigated the 118 A>G SNP in regard to μ-opioid receptor signalling, expression and binding affinity in human brain tissues, sampled post mortem from two regions involved in the sensory processing of nociceptive information. The main effect of the 118 A>G SNP was shown to be a reduction of the agonist-induced receptor signalling efficacy, and these results were only evident in the secondary somatosensory area (S_{11}) of the brain (Oertel, Kettner et al. 2008).

5.1.4 The frequency of the variant G allele

Four patients were homozygous GG for the 118A>G SNP in our study. The relatively low frequency of occurrence of the SNP brings a limitation to studies with small sample sizes. In many of the above mentioned studies the number of homozygous GG carriers of the 118 A>G SNP are low, with values varying from zero to 10 in the

clinical studies (Chou, Wang et al. 2006; Chou, Yang et al. 2006; Coulbault, Beaussier et al. 2006; Campa, Gioia et al. 2008; Landau, Kern et al. 2008) and values varying from zero to six in the studies including healthy volunteers (Lotsch, Skarke et al. 2002; Skarke, Darimont et al. 2003; Romberg, Olofsen et al. 2005; Lotsch, Skarke et al. 2006; Oertel, Schmidt et al. 2006). One exception is the study by Sia *et al*., where the numbers of GG individuals are 82 (Sia, Lim et al. 2008). Future studies should involve large sample sizes to elucidate the role of the 118A>G SNP in the *OPRM1* gene and opioid efficacy.

5.2 The COMT enzyme (Paper II and IV)

Paper II and IV show that the variability in the *COMT* gene influences on morphine requirements in a cancer patient cohort. Paper II answers the research question number two, and suggests that the rs4680 SNP is influencing the efficacy of morphine. Patients homozygous Met/Met needed less morphine (95±99 mg/24h) than homozygous Val/Val (155±160 mg/24h) patients. Paper IV answers the research question number four, and suggests that more of the genetic variability in the *COMT* gene is influencing the efficacy of morphine in cancer patients with pain, by showing that carriers of a frequent haplotype defined by 11 SNPs across the entire *COMT* gene needed lower morphine doses than patients not carrying the haplotype. Median morphine doses for carriers of this haplotype were 60 mg/24h versus median morphine doses of 100 mg/24h for non-carriers of the haplotype. The available literature today on variability of the *COMT* gene and different pain phenotypes as well as opioid response is complex, in the sense that some studies find association to the well-studied rs4680 SNP (Zubieta, Heitzeg et al. 2003; Diatchenko, Nackley et al. 2006), whereas others do not (Kim, Neubert et al. 2004; Diatchenko, Nackley et al. 2006; Ross, Riley et al. 2008). Most studies have addressed the variability in the *COMT* gene with different aspects of pain sensitivity.

5.2.1 Variability in the *COMT* gene and pain perception

The study by Diatchenko *et al*., (Diatchenko, Slade et al. 2005), showing that haplotypes of the *COMT* gene were strongly associated to sensitivity of pain, has been followed up in a study using the same cohort, hypothesizing that different genetic

variants of the *COMT* gene contribute differently to human perception of different pain stimuli (Diatchenko, Nackley et al. 2006). The authors report that *COMT* diplotypes were strongest associated to thermal pain, and that the rate of temporal summation of heat pain did not differ among haplotype combinations. In contrast, the common and well-studied rs4680 SNP was associated to the rate of temporal summation of heat pain (a process mediated by the central nervous system), but not with other pain measures. Based on the results from this study, Diatchenko and co-workers suggest that the rs4680 SNP in the *COMT* gene may play a primary role in variation in temporal summation of heat pain, but that other SNPs of *COMT* haplotype exert a greater influence on resting nocieptive sensitivity (Diatchenko, Nackley et al. 2006). Furthermore, they emphasize the importance of this finding, arguing that these assumptions are consistent with two other studies investigating the rs4680 impact on pain perception (Zubieta, Heitzeg et al. 2003; Kim, Neubert et al. 2004). Kim *et al*., (Kim, Neubert et al. 2004), report no association between the rs4680 SNP and measures of pain sensitivity to transient noxious thermal stimuli (resting nociceptive sensitivity), while Zubieta *et al*., (Zubieta, Heitzeg et al. 2003) report an association between the SNP and muscle pain sensitivity evoked by prolonged infusion of hypertonic saline (a temporal integration of painful stimuli involving the central nervous system). Whereas Diatchenko and co-workers define haplotypes of the *COMT* gene as high pain sensitivity (HPS), average pain sensitivity (APS) and low pain sensitivity (LPS) haplotypes (Diatchenko, Slade et al. 2005), Kim and colleagues failed to see any association with haplotypes of the *COMT* gene addressing postsurgical pain. Instead they reported an association between a SNP (rs740603) located in intron 1 of the *COMT* gene with post-operative pain ratings (Kim, Lee et al. 2006). The complexity of the matter grows even more apparent when considering a recent study of patients undergoing arthroscopic shoulder surgery, where the findings of Diatchenko are supported (George, Wallace et al. 2008). In this study *COMT* diplotypes (APS/HPS) were associated with higher pre-operative ratings and increased risk of experiencing persistent pain following surgery (George, Wallace et al. 2008).

In summary, the available evidence is sufficient to conclude that COMT does in fact play a role in pain modulation, but it may take time to fully delineate that role (Lacroix-Fralish and Mogil 2008; Skorpen, Laugsand et al. 2008).

5.2.2 Variability in the *COMT* gene and opioid responses

Only a few studies have been carried out in clinical cohorts addressing COMT and responses to opioids. In fact, only cancer patients and their response to opioids during cancer pain treatment have been investigated. Three studies in this thesis (Paper II-IV), address the *COMT* gene as a likely contributor to the efficacy of morphine in a cancer patient cohort with pain. The first study investigating the *COMT* gene (Paper II) suggests that one SNP, the rs4680, influences the opioid efficacy in the treatment of cancer pain. The following studies (Paper III and IV) show and discuss a more complex genetic contribution from the *COMT* gene to the efficacy of opioids. A study by Ross and co-workers addressing COMT and central side effects in a cancer patient cohort, showed that *COMT* genotypes and a haplotype were associated to drowsiness and confusion or hallucinations (Ross, Riley et al. 2008). In contrast to the results from the papers addressing COMT in this thesis, morphine doses did not differ between genotypes or haplotypes of *COMT* in the study by Ross and co-workers. Patient cohorts in all studies mentioned in this chapter are of similar size, counting approximately 200 patients. To elucidate the role of COMT and the variability to opioid responses, larger sample sized data should be approached.

5.2.3 Haplotype construction

Knowledge of an individual's haplotype is important, given that individual SNPs usually influence with a minor contribution to biological functions. Haplotype construction enables us to study the overall effect of SNPs and haplotype analyses are more powerful tool for studying genetic association to disease or drug response. In paper IV we have chosen to construct and present long haplotypes across the entire *COMT* gene. An alternative approach would have been to construct haplotypes defined by haploblocks in the gene. Haploblocks are regions in the gene where values of the linkage disequilibrium (LD) between SNPs are high (see 1.4 Genetic terms). Analyses of LD between SNPs have so far revealed three haploblocks in the *COMT* gene in

cohorts with European ancestry (Diatchenko, Slade et al. 2005; Kim, Lee et al. 2006). The long haplotype is defined by the total number of SNPs analysed and is therefore a combination of "haploblock-haplotypes" carried on one chromosome. The haplotypes defined by haploblocks can be looked upon as bricks in a jigsaw puzzle, but it is only when you manage to put the bricks together that you are able to see the whole "picture" in the jigsaw puzzle. Therefore, long haplotypes give extra genetic information in genetic analyses and to the individuals' genetic makeup. However, whether genetic analyses should select primary outcome of long haplotypes across genes, or rather shorter haplotypes constructed from haploblocks, is a question that needs to be debated. In this thesis we chose to present long haplotypes of the *COMT* gene only. Further data analyses elucidating the linkage disequilibrium between SNP and presentation of haploblock may have been appropriate. However, we would still have been forced to choose a primary outcome of the presentation and analyses of genetic groups, to protect against multiplicity of testing.

5.3 Combined effects of genes and opioid efficacy (Paper III)

Paper III answers the aim of study number three, and investigated the combined effects from two SNPs in the *OPRM1* and *COMT* genes. The SNPs, the 118A>G and the rs4680, have been suggested in previous papers (Paper I and II) to have a possible effect on opioid efficacy. We found that patients being homozygous A/A and Met/Met in the *OPRM1* and *COMT* genes, respectively, needed less morphine dose for pain relief than other patient groups with other allele combinations of the genes. One other study has addressed the gene joint effect of two SNPs in two genes relevant for opioid efficacy in a cancer patient cohort (Campa, Gioia et al. 2008), so studies addressing possible joint gene effects for the phenotypes of opioid response are (to my knowledge) at present only two. While we were addressing joint effects of the *OPRM1* and *COMT* genes, Campa *et al*., investigated joint effects of the C3435T SNP in the ABCB1/MDR1 gene and the 118 A>G SNP in the *OPRM1* gene. Combining the extreme genotypes in these genes, Campa and co-workers detected and defined three patient groups: Strong responders, responders and non-responders of morphine in a cancer patient cohort (Campa, Gioia et al. 2008).

Epistasis, the interaction between genes, is of major interest in molecular genetics. Most researchers today hypothesize that epistasis is an ubiquitous component of the genetic architecture, and that it is consequently more important to investigate the interactions between genes than the independent effects of any possible susceptibility gene (Moore 2003). Most likely, the most important elements for describing the phenotype of opioid response are interaction of susceptible genes together with important contribution of environmental factors. This plausible complex relation of epistasis and environment to opioid response is illustrated in Figure 7. The fact that only two studies at present have investigated the combined effect of genes on opioid response, demonstrates the scarcity of current research findings. Detection of many more genes and biological pathways will obviously be of utmost importance in future research.

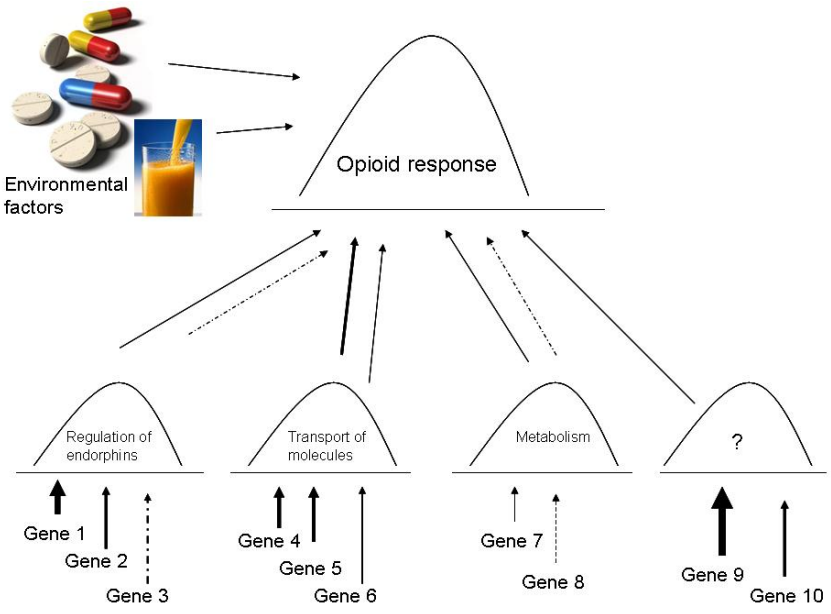

Figure 7: The relation of epistasis and environmental factors to opioid response. The different sizes of the arrows indicate that genes have different effect on the response of opioids. The question mark indicates that there are still unknown biological pathways that contribute to the response of opioids.

The joint effects of genes can be expected to have a synergistic (enhanced), antagonistic (suppressed) or no effect on the phenotype investigated. If epistasis is detected, it is often followed by an assumption that it can tell us something about the mechanisms and pathways involved in the phenotype investigated, in particular in relation to the biological interaction between the implicated proteins (Cordell 2002). Any given data pattern from the combined effect of two genes can be obtained from a number of different underlying biological mechanisms. Therefore, definite conclusions regarding synergistic and antagonistic effects are generally beyond the grasp of an association study (Thompson 1991). However, a careful consideration and discussion on possible biological mechanisms behind the suggested interaction of the *OPRM1* and the *COMT* genes is interesting, and may also bring forward hypotheses suited for research in the future. Based on the findings of Zubieta and co-workers (Zubieta, Heitzeg et al. 2003), we speculate that the contribution from the genetic variability in the *COMT* gene to the efficacy of morphine is due to a downregulation of μ-opioid receptors in various regions of the brain. However, experimental studies, such as in vitro cell studies and animal studies are needed to elucidate the role of interaction between genes.

The number of classifiable genetic subgroups increases when investigating joint effects of polymorphisms in multiple genes. The number of genetic subgroups derived from a study investigating two SNPs in two genes is nine. However, in Paper III only four genetic groups were created and this was done on the basis of the earlier findings that the *OPRM1* A/A genotype (Paper I) and the *COMT* Met/Met genotype (Paper II) required the least morphine doses. Carriers of the *COMT* Met/Met and the *OPRM1* A/A genotype were defined as one "true" genetic group, while other genetic subgroups were merged into one. For example, Met/Met and A/G carriers were merged into one genetic group together with Met/Met and G/G carriers, defined as Met/Met but not A/A carriers. Defining such merged genetic groups may be a limitation to the study since we do not know the contribution of the different genotypes to the possible interaction between them. The preliminary findings in Paper III reveal the importance of assessing

joint effects of genes, but there is a definite urge for larger samples sizes in future studies.

In Paper III the mean morphine dose (117mg/24h) was used to divide groups of low morphine dose users (=<117mg/24h) and high morphine dose users (>117mg/24h). A question can be raised whether this is the appropriate cut-off between low dose and high dose users. A better design would perhaps have been to use 25 % of the patients with the lowest doses of morphine and 25 % of the patients with the highest doses of morphine. This procedure would have gained a more extreme phenotype, but would have reduced the sample size by 50%. Statistical power decreases when sample sizes are reduced, followed by the probability of not detecting a statistical significant association even if it is present. The importance of measuring the "correct" phenotype, however, should always be a priority taken into consideration the simultaneous wish for a large sample study.

5.4 A balanced view of association studies

Genetic association studies have been the main tool and an important approach in the effort to indentify candidate genes and genetic variation underlying the efficacy of opioids. The objective of an association study is to seek a statistical association between genetic variant(s) and a phenotypic variation. The development of genetic medicine the last decades (as illustrated in Figure 1 in the introduction) has made it possible to test for more than one million genetic variants in a single experiment. Although genotyping technology and genome-wide approaches are available today, it has been here for only a short period of time (Iles 2008). To my knowledge the current association studies addressing the efficacy of opioids in humans have exclusively used a candidate-gene approach, in which one or several genes – hypothesized to be involved in the biology of opioid action – are studied. Reviewed in Skorpen and Laugsand, a total of 15 candidate genes have been investigated in the search for genetic variation contributing to μ-opioid responses (Skorpen, Laugsand et al. 2008).

Despite early initial optimism for genetic association studies (Risch and Merikangas 1996), the merit of such studies is now strongly debated (Hirschhorn, Lohmueller et al. 2002; Ioannidis 2003; Lohmueller, Pearce et al. 2003; Page, George et al. 2003; Moonesinghe, Khoury et al. 2007) because of the high proportion of false positive findings (Ioannidis 2005). A comprehensive review, carried out in 2002, performed a survey of more than 600 positive associations between common gene variants and disease. Out of 166 reported associations studied three or more times, only six were replicated consistently (Hirschhorn, Lohmueller et al. 2002).

The purpose of a replication study is to evaluate a positive (or negative) finding from a previous study, in order to provide credibility that the initial findings are valid. More specifically, a replication study provides insurance against biases and errors (that can unavoidably afflict any study), among others inappropriate control groups, investigator biases, over-elaborate data exploration, and genotyping errors (Page, George et al. 2003). A quality assessment of genetic association studies supporting susceptibility for acute lung injury has been carried out by Flores and co-workers (Flores, Pino-Yanes Mdel et al. 2008). This study investigated 14 criteria, based on a checklist suggested by the NCI-NHGRI Working Group on Replication in Association Studies (Chanock, Manolio et al. 2007), and scored these criteria as 1 if present and 0 if absent. Of 16 reported genes associated to acute lung injury, four genes were the most replicated across studies, and the studies on average had an intermediate quality score. Attempts to assess the quality of the current genetic association studies are of crucial importance to the validation of results, but have to my knowledge not been carried through addressing pharmacogenetic studies of opioids.

Liu *et al*., argue that the research field of genomics may have unreasonable high expectations of success of replication, and question whether it is sufficient or necessary to treat replication as the gold standard for defining true variants (Liu, Papasian et al. 2008). Functional studies, such as gene expression studies at the RNA level and proteomics studies at the protein level, may provide useful and complementary information to genetic association studies as they can support (or not support) findings

in studies on the DNA level. In other words, such studies may reveal information on how the gene(s) contribute to the trait investigated.

In conclusion, a single association study can be considered as a stage on a journey that starts with ignorance and hopefully ends with a clear and robust conclusion on whether a given gene locus contributes to the trait investigated (Hattersley and McCarthy 2005). However, time has come to be more stringent and mature when planning a design for an association study. Reasonable criteria for future association studies include the use of low P-values, large sample sizes, replication in multiple samples, and avoidance of population stratification. Meta-analyses and functional studies are also of importance and ideal to guide interpretation of findings from genetic association studies.

5.5 Unequivocal genotype and phenotype?

5.5.1 Is the genotype correct?

SNPs are the most frequent genetic variation in genes, but there are also other variations within the DNA sequence, such as microsatellites, short tandem repeats (STRs), and insertions and deletions, all of which may have an impact on complex phenotypes. DNA methylation, modification of histones and chromatin, and RNA interference represent examples of epigenetics and have been suggested to have a mechanism of biological heredity, even though these variations are not based on variation in the DNA sequence (Pembrey 2002). Recent findings, including copy number variation (CNV) (Freeman, Perry et al. 2006; Locke, Sharp et al. 2006; McEwen, Woolfe et al. 2006), short transcripts of unknown function (Gingeras 2007), and gene-gene interactions describe even more of the complexity in the human genome. All the above mentioned genetic variation may mask the effect of any given SNP on a complex phenotype. New discoveries of the human genome clearly indicate that a genotype of an individual, earlier coined as “an assignment by scientific investigators without any room for error”, is more uncertain than expected. Thus, genotype errors are most likely present in the majority of published association studies. The term *unequivocal genotype* is replaced by *equivocal genotype,* and more than three dozen factors causing the uncertainty of a genotype have been reported (Nebert, Zhang et al. 2008). However, analysis of SNPs will continue to be of considerable importance in

large-scale genome wide association studies (GWAS) and in large-scale replication studies, but a careful discussion and interpretation of the SNPs contribution to the complex biological pathway is needed.

5.5.2 Genetic diversity and ethnic considerations

In the late 1990s, the term *single nucleotide polymorphism* (SNP) was defined, formerly known as "nucleotide substitution" (Nebert, Zhang et al. 2008). With the concurrent improvements in the technology for SNP detection at that time, hundreds of publications in the years that followed sought to find associations between one or a few SNPs and a multiplex phenotype. SNPs are the most frequent genetic variation in genes. At present the number of SNPs in the human genome, released in National Center for Biotechnology Information (NCBI) dbSNP Build 129 (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi), is nearly 15 million, and more than 6 million of theses are validated. Considering only the 6 million validated SNPs in the ~ 3 billion base pair long human genome, the rate of SNPs is 1 per 500 base pair. Only 1.1 % of the genome is spanned by exons, 24 % by introns, and 75 % of the genome is intergenic DNA (Venter, Adams et al. 2001). Even though any two human individuals are more than 99% identical to each other on the basis of DNA comparisons, there are many ethnical differences that need to be carefully considered in genetic association studies. For example, the rs4680 (Val158Met) polymorphism in the *COMT* gene is reported to vary from 0.01 to 0.62 for the Met allele in a global sample population (Palmatier, Kang et al. 1999). Also, despite the relative low genetic differentiation among Europeans, a subtle population structure has been discovered where genetic variation mirrors the geography of Europe, and an individual's DNA can be used to conclude on the individual's origin with surprising accuracy (Novembre, Johnson et al. 2008).

Another important issue of genetic variability is that SNPs are only one type of genetic variation. As mentioned, structural variations of the genome, such as CNVs and balanced chromosomal rearrangements (inversions) have gained much attention in the field of human genetics. CNVs are recognized to contribute to a major proportion of the

genetic differences in humans and estimated to account for more of the differences seen in any two individuals than the differences due to SNPs (Sebat 2007). Global variation in CPV has been reported for populations with ancestry in Europe, Africa and Asia (Redon, Ishikawa et al. 2006), so awareness for population stratification is needed also for CPV studies.

To summarize, the past few years have shown that the variability in the human genome is larger then earlier appreciated (Sebat 2007) and the differences between ethnical groups are more apparent and more specific than previously known (Novembre, Johnson et al. 2008). Therefore, it is important to precisely state the ethnical and geographical origin of populations in genetic association studies.

5.5.3 Accuracy in measuring phenotype

Clear clinical descriptions are necessary to define a phenotype unequivocally. In a palliative cancer patient cohort treated with opioids, the potential phenotype may be any particular response seen after opioid administration. In Paper I, II and IV the continuous variable "24 hour morphine dose" is chosen to represent the phenotype "morphine requirements to relieve cancer pain", and in Paper III we divide the patient population into groups of high dose morphine users (>117mg/24h) and low dose morphine users (<117mg/24h). It can be discussed whether these phenotype definitions in the presented papers are unequivocal and "tell the truth" about the requirements of morphine for the cancer patients. Taking into consideration the complexity of clinical medicine, and even more so the area of pain assessment, it is most likely that there are factors in our studies contributing to the lack of an unequivocal phenotype. For instance it is evident that the phenotype "pain" may contribute to the need for morphine. Unfortunately, some patients in our cohort report too much pain to be considered adequately treated with morphine. In Paper I we chose to include only patients adequately treated (inclusion criteria being average pain reporting less or equal to 4), to protect against the possible "noise" from patients not receiving sufficient doses of morphine for their cancer pain. Despite this attempt to make the patient group more homogenous, the reported pain was differently distributed between genotype groups

(1.9±1.5, 3.1±1.1 and 2.0±1.2 for AA, AG and GG genotype groups respectively), illustrating the need to discuss and carefully consider possibly confounding factors to the phenotype investigated. In general, confounding factors are seldom easily measured, and attempts to protect against them pre study are difficult. Considering the palliative cancer patient cohort in this thesis, the effect of opioids can be "hidden" or misinterpreted because other cancer disease symptoms influence the patients' assessment of the pain treatment. Is vomiting and nausea caused by the cancer disease or the opioid administration? Is a possible untreated depression causing an effect on opioid response? We know that there are interactions between other medications and opioids, but to which extent is often unknown. More than 18 factors that can cause an equivocal phenotype have been suggested (Nebert and Vesell 2004). There will clearly be an ever existing challenge to define a "precise" phenotype, and a discussion on potential factors' contribution to the lack of unambiguity in a pharmacogenetic study is definitely needed.

6. Conclusion and future perspectives

We have investigated two genes and their association to opioid efficacy. Based on our findings and the supplementary recent literature, we suggest that both the *COMT* and the *OPRM1* genes are important contributors to the analgesic effect of morphine. The present thesis can be viewed as the first step on the journey to elucidate genetic variations that contribute to opioid efficacy in cancer patients.

The current literature on the pharmacogenetics of opioids is limited to well-known polymorphisms in clear candidate genes, and sample sizes of typically a few to a couple of hundreds of individuals (Skorpen, Laugsand et al. 2008). A natural approach in the future will be to seek supporting evidence in larger sample sizes, and search for other genes that influence on opioid efficacy. A number of different study designs, such as family studies, twin studies, and studies in animals, may be applied to identify new genes to the list of contributors to opioid response, but genome wide association studies (GWAS) are the most likely approach for future studies. A collaboration project with 11 European countries (EPOS study initialized by our research team) is about to be finalized (March 2009) giving a biobank of pharmacological, genetic and clinical data of approximately 2300 cancer patients receiving opioids, mostly morphine and oxycodon, for pain treatment. The next steps will be to further investigate the enlarged cohort of cancer patients in the EPOS collaboration project with a genome-wide approach.

In principle, complex phenotypes such as opioid response might be more susceptible to "soft" forms of genetic variation, such as variation in non-coding sequences and copy number, which alter gene dose without abolishing gene function (McCarroll and Altshuler 2007). The genetic variation that contributes to the complex phenotype of opioid response is likely to be influenced by non-coding sequences and structural CNV of the DNA. To date there are no studies investigating variations of CNV and the response to opioids. Future studies addressing CNV and the efficacy of opioids for cancer pain will be interesting.

In the presented thesis the patient population was treated with morphine for cancer pain. Even though morphine is the opioid of choice for moderate to severe cancer pain, alternative opioids, such as oxycodon is becoming more frequently used in palliative cancer patients. The effect of different opioids may depend on different sets of genes, so studies addressing the use of different opioids need to be carried out in the future.

Most cancer patients experience several symptoms that may prevent and/or limit the degree of analgesic effect of opioids. Adverse effects such as sedation, nausea and vomiting, and euphoria are examples of symptoms that may have a genetic basis. Studies mapping the genetic variations influencing the interindividual differences in symptom occurrence have just begun, combining large cohort (EPOS study) and genome-wide association approaches.

A doctoral thesis carried through at our research team has validated the pain assessments commonly used in palliative care (Hølen 2008). One conclusion from this thesis is that better assessment tools for pain measurements are needed (Holen, Hjermstad et al. 2006). For self-reported pain assessment, our research team has in a collaboration project with the EAPC research network and with an EU grant, started the development of a new and improved computer based assessment tool. There are also strong initiatives within the palliative care community that there is a need for developing an international consensus on how to classify pain in cancer patients (Kaasa and Radbruch 2008; Knudsen, Aass et al. 2009).

Taking into consideration the lack of stringent criteria in published association studies and the difficulties defining unequivocal traits and genetic groups, it remains unclear whether individualized drug therapy will be achievable by means of DNA testing alone (Nebert, Zhang et al. 2008). It is yet too early to arrive at a conclusion about how (and when) the “bench research” of genetics approaches the clinic and the patient’s “bedside”. However, there is no doubt that translational research will have advantages, and is the best approach in future studies.

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Paper I

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Paper II

Pain 116 (2005) 73–78

PAIN

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The Val158Met polymorphism of the human catechol-*O*-methyltransferase (COMT) gene may influence morphine requirements in cancer pain patients

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Received 16 December 2004; received in revised form 7 March 2005; accepted 29 March 2005

Abstract

Catechol-*O*-methyltransferase (COMT) inactivates dopamine, epinephrine and norepinephrine in the nervous system. A common functional polymorphism (Val158Met) leads to a three- to-four-fold variation in the COMT enzyme activity, the Met form displaying lower enzymatic activity. The Val158Met polymorphism affects pain perception, and subjects with the Met/Met genotype have the most pronounced response to experimental pain. Based on this information we analyzed the influence from the COMT Val158Met polymorphism on the efficacy of morphine in a cohort of patients suffering from cancer pain. We genotyped 207 Caucasian cancer patients on morphine treatment with respect to the Val158Met polymorphism and compared the morphine doses, serum concentrations of morphine and morphine metabolites between the genotype groups. Patients with the Val/Val genotype ($n=44$) needed more morphine (155 ± 160 mg/24 h) when compared to the Val/Met (117 ± 100 mg/24 h; $n=96$) and the Met/Met genotype (95 ± 99 mg/24 h; $n=67$) groups ($P=0.025$). This difference was not explained by other factors such as duration of morphine treatment, performance status, time since diagnosis, perceived pain intensity, adverse symptoms, or time until death. These results suggest that genetic variation in the *COMT* gene may contribute to variability in the efficacy of morphine in cancer pain treatment.

Keywords: Pain; Genotyping; Genetic variation; Opioid

1. Introduction

The catechol-*O*-methyltransferase (COMT) enzyme metabolizes the catecholamines dopamine, epinephrine and norepinephrine, and is a key modulator of dopaminergic and adrenergic neurotransmission. The involvement of catecholamines in pain modulation is known from clinical (Ali et al., 2000; Niemi and Breivik, 2002) and experimental studies (Bie et al., 2003; Raja et al., 1995; Sagen and Proudfit, 1985). It has been shown in animal models that the neuronal content of enkephalins is reduced by chronic activation of dopaminergic neurotransmission (Steiner and Gerfen, 1998). An upregulation of μ opioid receptors in various regions of the brain follows the reduction of enkephalin content (Chen et al., 1993; Steiner and Gerfen, 1998). Based on this knowledge, Zubieta et al. (2003) investigated the relationship between the common polymorphism Val158Met in the *COMT* gene and pain perception and opioid receptor density in volunteers. The Val158Met polymorphism causes a valine (Val) to methionine (Met) substitution at codon 158 in the COMT

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0304-3959/$20.00
doi:10.1016/j.pain.2005.03.032

enzyme, leading to a three- to four-fold reduced activity of the enzyme (Lachman et al., 1996; Lotta et al., 1995). Therefore, the Val/Val, Val/Met and Met/Met genotypes may predict a high, intermediate and low COMT enzyme activity, respectively. Zubieta et al. (2003) hypothesized that enhanced activation of the dopaminergic neurotransmission in individuals with the low-activity COMT enzyme would result in lower levels of enkephalins, and consequently more pain due to decreased endogenous opioid analgesia. They confirmed that individuals with the COMT Met/Met genotype had higher sensory and affective ratings of pain following an experimental pain stimulus, and also that individuals with the Met/Met genotype had higher regional density of μ opioid receptors (Zubieta et al., 2003).

Morphine pharmacology is complex and may be influenced by genetic variation related to morphine metabolism, opioid receptors and blood–brain-barrier transport (Duguay et al., 2004; Klepstad et al., 2004; Thompson et al., 2000). We have previously reported that the 118 A>G polymorphism in the μ opioid receptor gene increases morphine requirements in patients with pain caused by malignant disease (Klepstad et al., 2004). Opioid efficacy may also be affected by genetic variation in non-opioid biological systems interacting with opioids. Thus, the experimental findings in the study by Zubieta et al. (2003) prompted us to investigate whether the COMT Val158Met polymorphism influences pain in a clinical setting. The effect of the polymorphism could be hypothesized to be bidirectional. The patients with the Met/Met genotype have lower neuronal content of enkephalin, which might lead to decreased endogenous analgesia and a compensatory requirement for higher morphine doses. Alternatively, the demonstrated increase of μ opioid receptors might lead to an increased effect from morphine administration in Met/Met genotype individuals. Based upon these hypotheses we analyzed the influence from the COMT Val158Met polymorphism on the efficacy of morphine in a population suffering from cancer pain.

2. Materials and methods

2.1. Ethics

The study was carried out in accordance to the principles of the Helsinki declaration. The Regional Committee for Medical Research Ethics, Health Region IV, Norway, approved the study, and all patients gave their oral and written informed consent before inclusion in the study.

2.2. Subjects

We analyzed a cancer patient population included (from June 1999 to February 2000) in a study originally designed for assessing the relationship between patients' characteristics and serum concentrations of morphine and metabolites (Klepstad et al., 2003). All patients were Caucasians and in-patients at the 900-bed tertiary St Olav University Hospital in Trondheim, Norway, receiving scheduled oral morphine for cancer pain treatment.

The following information was collected from the hospital records for each patient: age, gender, ethnicity, cancer diagnosis, time since diagnosis and time since start of morphine. The daily morphine doses were collected from the patients' ward charts. Survival times from the time of inclusion were obtained from the death records of Norway.

2.3. Assessments

Pain was measured using the item 'average pain' during the last 24 h in the Brief Pain Inventory (BPI) questionnaire. The patients rated pain on an 11-point numeric scale, where 0 represents 'no pain' and 10 represents 'pain as bad as you can imagine'. The BPI is developed for the use in cancer pain patients, validated in Norwegian, and recommended by the European Association of Palliative Care for use in clinical studies (Caraceni et al., 2002; Daut et al., 1983; Klepstad et al., 2002). The European Organization for Research and Treatment of Cancer core quality-of-life questionnaire (EORTC QLQ-C30) version 3.0 was used to assess the patients' nausea/vomiting, constipation, fatigue and tiredness (Aaronson et al., 1993). Cognitive function was assessed with the Mini Mental State (MMS) examination. The MMS score ranges from 0 to 30, higher scores meaning better cognitive function (Folstein et al., 1975). The patients' functional status was assessed by the Karnofsky performance status (Karnofsky et al., 1948).

2.4. Blood samples and pharmacogenetic analyses

Collection of blood samples and determination of serum concentration of morphine and its metabolites (morphine-6-glucuronide and morphine-3-glucuronide) were done as described in a previous work from our group (Klepstad et al., 2003). Genomic DNA was isolated from 50 to 200 μl EDTA blood on a MagNA Pure LC (Roche Diagnostics Scandinavia AB, Bromma, Sweden) using the MagNA Pure LC DNA Isolation Kit I applying the manufacturers high performance protocol. Purified genomic DNA was eluted in 100 μl elution buffer and stored at −20 °C.

COMT genotypes were determined using the LightCycler (Roche Diagnostics Scandinavia AB, Bromma, Sweden) fluorescence resonance energy transfer method (Wittwer et al., 1997). Polymerase chain reaction (PCR) amplifications were performed in 20 μl reactions on a LightCycler System, using 2 μl purified genomic DNA and the LightCycler-FastStart DNA Master Hybridization Probes kit (Roche Diagnostics Scandinavia AB, Bromma, Sweden). PCR primers (Eurogentec, Seraing, Belgium)

Table 1
Primers and hybridization probes used for Val158Met genotyping

	Sequence
Primers	Forward 5′-ACGCCGTGATTCAGGAGCA-3′ Reverse 5′-GTCTTTCCTCAGCCCCAG-3′
Probes	Sensor 5′-TCA**C**GCCAGCGAAATCCA-Fl-3′ Anchor 5′ LC Red 640-ATCCGCTGGGT-GATGGCG-3′

*Fl, fluorescein; LC Red 640, Light Cycler Red 640. Bold **C** indicates polymorphic site.

Table 2
Amplification and melting curve conditions for COMT genotypes

PCR cycling conditions				Melting curve conditions	
Denaturation	Annealing	Extension	Cycles	Stepwise temp. decrease	Temp. increase
95 °C for 15 s	62 °C for 15 s	72 °C for 14 s	10	90 °C for 15 s; 66 °C, 58 °C and 40 °C for 20 s each	From 40 to 90 °C at 0.4 °C/s
95 °C for 15 s	60 °C for 15 s	72 °C for 14 s	14		
95 °C for 15 s	56 °C for 15 s	72 °C for 14 s	20		

Temp, temperature.

and fluorescence labeled probes (PROLIGO, Paris, France) are shown in Table 1. Conditions for PCR and melting curve analyses are shown in Table 2. The Val158Met polymorphism was verified using standard DNA sequencing methods as described previously (Holthe et al., 2003).

2.5. Statistical analysis

Descriptive data are given as mean (SD). Because the COMT alleles are expected to be codominant with respect to total COMT enzyme activity, we expected a gene-dose effect of the Val158Met polymorphism in respect to the need for morphine. In other words, we expected the morphine doses of the Val/Met heterozygous carriers to be in-between the morphine doses of the Val/Val and Met/Met homozygous carriers. Comparisons between the different genotypes were therefore performed with the Jonckheere–Terpstra test, a non-parametric test working with the hypothesis that $\mu_1 \leq \mu_2 \leq \mu_3$ (or the opposite $\mu_1 \geq \mu_2 \geq \mu_3$) (Hollander and Wolfe, 1999).

Post Hoc tests were performed with Mann–Withney *U*-tests. A *P*-value less than 0.05 was considered statistical significant. A prestudy formal sample size calculation was not performed since the data material and the number of patients originally was collected for a different study (Klepstad et al., 2003). The statistical software SPSS for Windows v. 11.5 was used for all statistical analyses.

3. Results

3.1. Patients

Two hundred and seven patients receiving chronic oral morphine treatment for cancer pain were included in the study. The patients' gender distribution, age, primary tumor locations, Karnofsky performance status, time since diagnosis, time since morphine treatment started and time from inclusion until death are shown for each genotype in Table 3. We observed no differences between the various genotype groups (Val/Val, Val/Met and Met/Met) for these patients' characteristics. We observed no differences in the pain intensity between the three genotype groups (Table 4). The intensities of other symptoms such as fatigue, nausea and vomiting, dyspnea, sleep disturbance, loss of appetite and constipation were similar between the three groups (Table 4). The patients' cognitive function measured by MMS sum score was similar between the groups (Table 4). We also observed no differences in renal and hepatic functions as assessed by serum creatinine concentrations and serum albumin concentrations between the three genotype groups (Table 3). All patients were Caucasians.

3.2. Genotype distributions

The observed genotype frequencies were 44, 96 and 67 for the Val/Val, Val/Met and Met/Met, respectively (Table 5). The relative frequency of the Met allele was 0.56, which is in the upper range of known allele frequencies in European populations (Biomed European Bipolar Collaborative Group, 1997). The Val158Met polymorphism distribution for the 207 cancer patients was in Hardy–Weinberg equilibrium ($\chi^2 = 0.767$; $P = 0.381$).

Table 3
Patient demographics for Val158Met genotype groups

	Val/Val ($n=44$)	Val/Met ($n=96$)	Met/Met ($n=67$)
Gender male: female	24:20	55:41	38:29
Age	65 (11)	64 (13)	61 (13)
Tumor diagnosis			
Urologic	10	32	17
Lung	8	17	14
Breast	9	20	16
Gastrointestinal	6	10	3
Others	11	17	17
Karnofsky performance status	67 (10)	65 (14)	70 (12)
Creatinine serum (μmol/l)	86 (34)	83 (25)	88 (40)
Albumin serum (g/l)	31 (5)	33 (6)	33 (5)
Time since diagnosis (months)	35 (49)	41 (50)	38 (47)
Time since morphine treatment started (months)	3.7 (6.1)	3.4 (6.1)	3.3 (8.4)
Survival time after study (months)[a]	5.5 (6.0)	4.9 (5.2)	5.8 (6.5)

All numbers are absolute numbers or mean (SD). No statistically significant differences were observed between groups. Categorical data were analyzed using the chi-square test, and continuous variables were evaluated with One-Way ANOVA if homogeneity tests were not significant. Kruskal Wallis test was used when homogeneity tests resulted in significant values.

[a] Survival times from the time of inclusion were obtain from the total number of patients with the Val/Val genotype, $n=41$; Val/Met genotype, $n=85$; and Met/Met genotype, $n=61$.

Table 4
Patient symptoms for Val158Met genotype groups

	Val/Val ($n=44$)	Val/Met ($n=96$)	Met/Met ($n=67$)
BPI average pain	3.9 (2.2)	3.7 (2.6)	3.5 (2.3)
Fatigue EORTC score	73 (23)	62 (24)	66 (22)
Nausea and vomiting EORTC score	30 (27)	24 (26)	29 (27)
Dyspnea EORTC score	39 (35)	38 (34)	32 (33)
Sleep EORTC score	35 (34)	34 (36)	35 (35)
Appetite EORTC score	64 (36)	49 (38)	53 (38)
Constipation EORTC score	56 (41)	57 (37)	54 (37)
Mini mental examination sum score	26 (4)	26 (3)	26 (4)

All numbers are mean (SD). No statistically significant differences were observed between groups (One-Way ANOVA). Kruskal Wallis test was used when homogeneity tests resulted in significant values.

3.3. Pharmacological observations

There were significant differences between the genotype groups when comparing morphine doses. Patients with the Val/Val genotype ($n=44$) received 155 ± 160 mg/24 h morphine, patients with the Val/Met genotype received 117 ± 100 mg/24 h ($n=96$) and patients with the Met/Met genotype received 95 ± 99 mg/24 h ($n=67$) (Jonkcheere–Terpstra test, $P=0.025$). Post Hoc Mann–Whitney tests showed that patients with the Val/Val genotype received significantly higher daily morphine doses when compared to patients with the Met/Met genotype ($P=0.03$), while the heterozygous Val/Met group did not reach statistically significant difference when compared to the other genotype groups (Table 6).

The observed mean serum concentrations of morphine, M6G and M3G were higher in patients homozygous Val/Val than in patients heterozygous Val/Met, and higher in patients heterozygous than in patients homozygous Met/Met. These observations did not reach statistically significant levels (Jonckheere–Terpstra test: morphine serum concentration, $P=0.85$; M6G serum concentration, $P=0.06$; M3G serum concentration, $P=0.14$) (Table 6). Because the Jonckheere–Terpstra tests did not indicate statistical significant differences as defined by a P-value >0.05, no post hoc tests were performed.

Table 5
COMT genotype and allele frequencies in the total of 207 cancer patients

	Genotype frequencies			Allele frequencies	
	Val/Val	Val/Met	Met/Met	Val	Met
N	44	96	67	184	230
Relative frequencies	0.21	0.47	0.32	0.44	0.56

Table 6
Pharmacological observations for Val158Met genotype groups

	Val/Val ($n=44$)	Val/Met ($n=96$)	Met/Met ($n=67$)
Morphine dose (mg/24 h)[a,b]	155 (160)	117 (100)	95 (99)
Morphine serum (nmol/l)	119 (199)	86 (88)	78 (72)
M6G serum (nmol/l)	711 (992)	506 (493)	410 (484)
M3G serum (nmol/l)	3809 (4436)	2812 (2209)	2536 (2707)

All numbers are mean (SD). No statistically significant differences were observed for the other observations ($P=0.06$ for differences in M6G conc.; $P=0.14$ for differences in M3G conc.; $P=0.85$ for differences in morphine conc.).

[a] (Jonkcheere–Terpstra test); $P=0.025$.

[b] Post Hoc Mann–Whitney tests; $P=0.03$ for differences between Val/Val and Met/Met genotype groups.

4. Discussion

We have addressed a possible contribution of the COMT Val158Met polymorphism to inter-individual variation in morphine requirements among patients suffering from cancer pain. The patients with the Val/Val genotype needed more morphine when compared to patients with the Met/Met genotype.

Several studies have shown that the dose of morphine required to achieve adequate pain control varies between patients. A three-fold variation in morphine doses has been observed among patients at similar stage of cancer pain (start of step III treatment at the WHO pain ladder) (Klepstad et al., 2000b). The inter-individual differences observed for serum concentrations of morphine and morphine metabolites are more pronounced than the inter-individual difference in morphine doses (Klepstad et al., 2000a). This observation indicates that variable pharmacokinetics is not the sole factor contributing to the variable need for morphine. Epidemiological observations suggest that genetic dispositions are important for this inter-individual variability in opioid pharmacodynamics as exemplified by that Native Americans have a more pronounced morphine induced depression of the ventilatory response compared with Caucasians (Cepeda et al., 2001), and Caucasians have shown to become more sedated and to exhibit a more depressed ventilatory response than Asians (Zhou et al., 1993).

The complexity of morphine pharmacology suggests that the variability in opioid pain treatment is associated with genetic variation in several genes. Genes that may influence the efficacy of morphine include the *UGT2B7* gene (Duguay et al., 2004), which product is responsible for morphine metabolism, the *OPRM1* gene encoding the μ opioid receptor (Klepstad et al., 2004; Lotsch et al., 2002), and the *MDR1* gene (Thompson et al., 2000) encoding the P-glycoprotein which may be involved in the transport of morphine across the blood–brain-barrier.

Non-opioid systems such as the adrenergic system may also influence on opioid analgesia. Both clinical (Ali et al., 2000; Niemi and Breivik, 2002) and experimental studies (Bie et al., 2003; Raja et al., 1995; Sagen and Proudfit, 1985)

have shown an improved analgesic effect of opioids with the concomitant use of catecholamines. COMT is the main enzyme metabolizing the catecholamines dopamine, epinephrine and norepinephrine. In vitro analysis of COMT enzyme activity has shown that the Val158Met polymorphism in the *COMT* gene affects the thermostability of the enzyme. The Met form is thermolabile, displaying a three- to four-fold reduced activity at 37 °C compared to the Val form of the enzyme (Lotta et al., 1995). However, the activity of the Met variant was normalized by cofactors like *S*-Adenosyl-L-methionine and magnesium (Lotta et al., 1995). The implications of a putative stabilizing effect of these compounds on COMT activity in vivo are not established.

It has been shown from animal studies that the neuronal content of enkephalins is reduced by chronic activation of dopaminergic neurotransmission (Steiner and Gerfen, 1998). The reduction of enkephalin content is followed by an up-regulation of μ opioid receptor density in various regions of the brain (Chen et al., 1993; Steiner and Gerfen, 1998). Variable COMT enzyme activity may alter dopaminergic activity and could therefore, through an altered action of dopaminergic substances, influence on the enkephalin content and opioid receptor density. Zubieta et al. (2003) observed that volunteers with the Met/Met genotype had a lower tolerance to pain and a higher regional μ opioid receptor density compared with individuals with Val/Met and Val/Val genotype. In the present study we observed that cancer patients with the Met/Met genotype needed lower doses of morphine in order to relieve pain compared with patients with the Val/Val genotype. This observation is intriguing as individuals with the Met/Met genotype also had low tolerance to pain (Zubieta et al., 2003). One possible explanation for our observation of lower morphine requirements in cancer pain patients with the Met/Met genotype could be that an increase of μ opioid receptor density causes morphine to be more effective in individuals carrying this genotype.

Opioid tolerance is well established in experimental models (Portenoy, 1994) but the significance of tolerance with respect to the efficacy of morphine during chronic cancer pain treatment is difficult to reproduce in clinical studies (Collin et al., 1993). Tolerance development with down-regulation of μ opioid receptors could be argued to counteract the increase in receptor density associated with the Met/Met COMT genotype. We collected data from a cohort of patients were the mean duration of morphine treatment in the three genotype groups ranged from 3.3 to 3.7 months, and there was no statistical difference between the genotype groups in respect to the duration of morphine treatment (Table 3). Hence, our results suggest that the influence of COMT variability on morphine need, if due to receptor density, is not confounded by the possible development of opioid tolerance.

We recognize some limitations in our study. Cancer patients represent a heterogeneous patient group prone to be influenced from several possible confounders. The severity of the disease and the nociceptive stimuli will vary between patients, patients might have variable organ function, and the treatment with other drugs can give rise to drug interactions. To check whether the genotype groups were different with regard to factors that might influence morphine requirements we compared the different genotype groups for several characteristics associated with the severity of the malignant disease such as time since diagnosis, time since morphine treatment started, performance status and time from study inclusion until death. The different genotype groups showed similar results for these characteristics as for other possible confounders including age, gender, renal function and liver function (Table 3). We chose a clinical research design in this study, including individuals receiving chronic morphine treatment for cancer pain. We believe that studies examining the effect of genetic variation in a clinical setting are important because they provide information that is not easily obtained in studies performed in controlled experimental settings. Simply stated, the clinical studies answer whether genetic variation is of clinical significance.

In this study we observed statistically significant differences in morphine doses between genotypes, but not for the serum concentrations of morphine and its metabolites. However, there was a trend towards an increase in serum concentration of M6G when comparing the genotype groups; the Met/Met, Val/Met and Val/Val genotypes displayed the lowest, intermediate and highest serum concentration, respectively. This trend is likely to reflect the same biological effect of the Val158Met polymorphism as seen with the morphine doses. In general, the inter-individual variation of serum concentrations is more pronounced than for the morphine doses, and consequently an increased number of patients are needed for an observed difference to reach statistical significance. In future studies a prestudy sample size calculation should be performed to calculate the sample size needed in order to compensate for the variability of serum concentrations and hence avoid the risk of a type II error.

Pain is a trait with a complex genetic basis, and most likely the efficacy of analgesics is influenced by a number of genes. Therefore, this study cannot determine the relative importance of the Val158Met polymorphism compared to other variations in genes that are likely to influence on the requirement of morphine. The results of the present study suggest that the Val158Met polymorphism in the *COMT* gene may contribute to the variability in clinical efficacy of morphine when administered to cancer pain patients. This finding is of principle interest since it demonstrates that genetic variability in genes relevant to non-opioid systems can influence the clinical efficacy of opioids.

Acknowledgements

The authors wish to thank the members of the 'Pain and Palliation Research Group' at the Faculty of Medicine,

NTNU, for valuable discussion. We would also like to thank Eirik Skogvoll, who participated in discussions on the statistical methods applied in this paper. This work was supported by grants from The Research Council of Norway, Norwegian University of Science and Technology, and the Central Norway Health Authority.

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Paper III

Pain 130 (2007) 25–30

PAIN

www.elsevier.com/locate/pain

Exploring joint effects of genes and the clinical efficacy of morphine for cancer pain: *OPRM1* and *COMT* gene

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Received 22 August 2006; received in revised form 6 October 2006; accepted 24 October 2006

Abstract

Pain is a complex human trait. It is likely that the interaction of multiple genes, each with a small individual effect, along with the effect of environmental factors, influences the clinical efficacy of opioids rather than a single gene alone. Polymorphisms in genes coding for the mu-opioid receptor (A118G) and catechol-*O*-methyl transferase (Val158Met) may be important modulators of opioid efficacy. We assessed joint effects of the OPRM1 and COMT genes in predicting morphine dose for cancer pain relief. We used genotype and clinical data from a pharmacokinetic study of morphine in 207 inpatients treated with stable morphine dose for at least 3 days by Palliative Medicine Specialists. Results showed significant variation in morphine dose requirement by genotype groups: carriers of COMT Val/Val and Val/Met genotype required 63% and 23%, respectively, higher morphine dose compared to carriers of Met/Met genotype ($p = 0.02$). Carriers of OPRM1 GG genotype required 93% higher morphine dose compared to carriers of AA genotypes ($p = 0.012$). When we explored for joint effects, we found that carriers of the OPRM1 AA and COMT Met/Met genotype required the lowest morphine dose to achieve pain relief (87 mg/24 h; 95%CI = 57,116) and those with neither Met/Met nor AA genotype needed the highest morphine dose (147 mg/24 h; 95%CI = 100,180). The significant joint effects for the Met/Met and AA genotypes ($p < 0.012$) persisted, even after controlling for demographic and clinical variables in the multivariable analyses. Future studies are needed to further characterize the joint effects of multiple genes, along with demographic and clinical variables, in predicting opioid dose.

Keywords: Cancer; Pain; Genetic; Epidemiology; Opioid; Joint effects

1. Introduction

Cancer pain is one of the most persistent and incapacitating symptoms of cancer. While opioids remain the drug of choice for cancer pain therapy (World Health Organisation Geneva, 1996) with morphine as the first line drug of choice, predicting the optimal morphine dose for patients remains a challenge. While traditionally, this inter-individual variability has been explained by differences in bioavailability, metabolism, differences in pain perception and other neurophysiological mechanisms, and socio cultural factors, evidence now suggests an important role of genetic variability in the clinical efficacy of opioids

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0304-3959/$32.00
doi:10.1016/j.pain.2006.10.023

(Pasternak, 2001; Lotsch et al., 2004; Klepstad et al., 2005).

As the most important target for morphine, polymorphisms of the gene for the mu opiod receptor (*OPRM1*) located on human chromosome 6q24-q25 (Wang et al., 1994), are primary candidates for genetic influences on the efficacy of opioids (Uhl et al., 1999; Hoehe et al., 2000; Koch et al., 2000; LaForge et al., 2000; Befort et al., 2001; Wang et al., 2001). Numerous single nucleotide polymorphisms (SNPs) in the *OPRM1* gene have been identified, but only a few have been explored for a possible relevance in opioid analgesia, including the A118G (Lotsch and Geisslinger, 2006b).

The influence of the polymorphic catechol-*O*-methyltransferase (*COMT*) gene located on chromosome 22 (22q11.21) to pain has been an active area of investigation. The Val158Met polymorphism, a common genetic variant, has been shown to influence the activity of the *COMT* enzyme. The enzyme, which metabolizes the catecholamines dopamine, epinephrine and norepinephrine, is also key modulator of dopaminergic and adrenergic neurotransmission.

Polymorphisms in genes coding for the *OPRM1* (Bond et al., 1998; Befort et al., 2001; Wang et al., 2001; Lotsch et al., 2002; Klepstad et al., 2004; Fillingim et al., 2005; Lotsch and Geisslinger, 2006b) and *COMT* (Lachman et al., 1996; Zubieta et al., 2003; Shield et al., 2004; Rakvag et al., 2005) may be important modulators of opioid efficacy. Pain is a complex human trait and it is likely that the interaction of multiple genes, each with a small individual effect, along with the effect of environmental factors, influences the clinical efficacy of opioids rather than a single gene alone.

The purpose of this study was to explore the joint effects of genes previously shown to have influence on the clinical efficacy of morphine in a sample of cancer patients receiving morphine treatment for cancer pain. We specifically assessed joint effects of variation in the *OPRM1* and the *COMT* genes in predicting morphine dose for pain control.

2. Patients and methods

We used data from the study of Klepstad et al. (2004) and Rakvag et al. (2005) that includes genotyping data and clinical variables for 207 patients admitted for cancer pain treatment. All patients were Caucasians and inpatients during the period June 1999 to February 2000 at St. Olav University Hospital, a 900-bed tertiary hospital in Trondheim, Norway. Patients were treated with stable morphine dose for at least 3 days before inclusion in the pharmacokinetic study of morphine. Patients aged <18, those not competent in the Norwegian language and those refusing consent to the study, were not included in the study.

Patients' hospital records were reviewed for age, gender, cancer diagnosis, time since diagnosis, presence of metastases and time since start of morphine. Clinical and laboratory variables including serum albumin and creatinine levels, and morphine dose for the last 24 h were abstracted from the patients' medical charts.

2.1. Pain assessment

Pain was measured using the item of 'average pain' during the last 24 h in the Brief Pain Inventory (BPI). The patients rated pain on an 11-point numeric scale, where 0 represents 'no pain' and 10 represents 'pain as bad as you can imagine'. Recommended by the European Association of Palliative Care (Caraceni et al., 2002) for use in clinical studies of pain, the BPI has been validated in Norwegian (Klepstad et al., 2002). The patients' functional status was assessed by the Karnofsky performance status (Yates et al., 1980; Mor et al., 1984).

2.2. Blood sample, DNA extraction and genotyping

Collection of blood samples was described previously (Klepstad et al., 2004). Briefly, genomic DNA was isolated from 50 to 200 ml EDTA blood on a MagNA Pure LC (Roche Diagnostics Scandinavia AB, Bromma, Sweden) using the MagNA Pure LC DNA Isolation Kit I applying the manufacturer's high performance protocol. Purified genomic DNA was eluted in 100 ml elution buffer and stored at K20 8C.

The procedure for genotyping was described previously (Klepstad et al., 2004; Rakvag et al., 2005).

2.3. Ethics

The study was conducted in accordance with the principles of the Helsinki Declaration and was approved by the Regional Committee for Medical Research Ethics, Health Region IV, Norway. Patients gave their oral and written informed consent before inclusion in the study.

2.3.1. Statistical analyses

We performed univariate comparisons of genotype frequencies using the χ^2 test. Comparisons across alleles for specific genotypes were performed using analysis of variance.

We conducted multivariable logistic regression analyses using morphine dose as the dependent variable. We used mean morphine dose (117 mg/24 h) to divide the groups (low = <117 mg/24 h; high > 117 mg/24 h). Given the exploratory nature of this study, we did not assume gene-dose effect and instead created dummy variables for the different genotypes. The first model included all the variables found significant at $p < 0.20$ in the univariate level of analysis. A p value of 0.20 was used as the cut-off since using a more traditional level ($p < 0.05$) often failed to identify variables known to be important (Bendel and Afifi, 1977). Further variable selection in the model was conducted by using backward elimination. With the goal of having the most parsimonious model, only variables with $p < 0.05$ were included in the final model. Colinearity diagnostics were also performed. The Statistical Package for Social Sciences was used in all the analyses (SPSS, 1998).

3. Results

Two hundred and seven patients, aged 29–89 years (mean age = 63), receiving chronic morphine treatment for cancer pain were included in this study. There were more males than females (56% versus 44%). The most common type of cancer was urologic (28%), followed by breast (22%) and lung (19%). Ninety percent of the sample had metastatic disease. Mean duration of morphine use was 3.4 months (SD = 6.9) and duration (time since cancer diagnosis) of cancer was from 0.4 to 50 months (mean = 24 months; SD = 48).

Patients received from 10 mg to 760 mg/24 h of morphine (mean = 117 mg/24 h; SD = 116 mg/24 h). The median pain score for the whole sample was 4. There were no statistically significant differences in pain intensity scores.

3.1. Genotype analyses

Allele frequencies and the results of the χ^2 test for separation from Hardy-Weinberg equilibrium showed that there was no significant departure from Hardy-Weinberg equilibrium for *COMT* Val158Met(23) and *OPRM1* A118G ($A = 0.888$; $G = 0.111$; $\chi^2 = 0.29$; $p = 0.91$).

The total morphine dose, median and mean pain intensity by polymorphisms (Panel A) and genotype combination (Panel B) are shown in Table 1. There were no statistically significant differences in pain scores across genotypes and joint genotype combination. Panel A shows that carriers of Val/Val and Val/Met genotype required 63% and 23%, respectively, higher morphine dose compared to carriers of Met/Met genotype ($p = 0.02$). For the OPRM1 gene, GG and AG genotype required 93% and 18%, respectively, higher morphine dose compared to carriers of AA genotypes [carriers of AA relative to GG ($p = 0.012$)].

Panel B shows the total morphine dose, median and mean pain intensity by joint genotype combination. Since carriers of the *COMT* Met/Met and the *OPRM1* AA genotypes required the least morphine dose, we created the following 4 groups: (1) Met/Met and AA; (2) Met/Met but not AA; (3) AA but not Met/Met; and (4) neither Met/Met nor AA. We observed statistical significance for the joint effects of Met/Met and AA genotypes ($p < 0.017$). We did not observe statistically significant differences for the other groups, which could be explained by the large variation in morphine dose for those groups.

3.2. Multivariable regression analyses

We assessed if the joint effects of *COMT* and *OPRM1* genotype on morphine dose will persist, controlling for variables known to potentially confound the relationship. Demographic variables such as age and gender and clinical variables such as Karnofsky status, time since cancer diagnosis and months using morphine and creatinine and albumin levels were included in the model. Variables found in the univariate analysis to be significantly associated with morphine dose at a level of $p < 0.20$ (Bendel and Afifi, 1977) were next entered into a multivariate logistic regression analysis with the purpose of building a model to determine the predictive

Table 1
Total morphine dose (mg/24 h) and pain outcomes by genotype (Panel A) and joint genotype combination (Panel B)

Genotype	Morphine dose mg/24 Mean	95%CI	Pain scores*** Pain intensity	
			Median	Mean (SD)
*Panel A**				
COMT				
Val/Val ($n = 44$)	155	106; 203	4	3.94 (2.2)
Val/Met ($n = 96$)	117	97; 137	3	3.66 (2.6)
Met/Met ($n = 67$)	95	71; 119	3.5	3.46 (2.3)
OPRM1				
AA ($n = 166$)	112	96; 128	3	3.60 (2.6)
AG ($n = 36$)	132	76; 187	4	4.10 (1.8)
GG ($n = 5$)	216	60; 371	2	2.0 (1.2)
*Panel B***				
Met/Met & AA ($n = 58$)	87	57; 116	3	3.18 (2.3)
AA but not Met/Met ($n = 108$)	126	104; 147	4	4.89 (1.8)
Met/Met but not AA ($n = 9$)	140	72; 224	5	3.83 (2.6)
Neither Met/Met nor AA (32)	147	100; 180	3	3.48 (1.8)

OPRM1, mu opioid receptor 1; COMT, cathechol-*O*-methyltransferase.

* Statistically significant difference for mean morphine dose for carriers of AA relative to GG ($p = 0.012$). Statistically significant difference for mean morphine dose for carriers of Val/Val relative Met/Met ($p = 0.023$).

** Statistically significant difference for mean morphine dose for carriers of Met/Met and AA relative to Neither Met/Met nor AA ($p = 0.017$).

*** No statistically significant differences for pain intensity scores by genotype groups.

value of genotype on morphine dose. Results showed joint Val158Met and A118G genotypes, months using morphine and time since cancer diagnosis as significant variables in predicting morphine dose. Table 2 shows that even after controlling for clinical variables, we observed statistical significance for the joint effects of *COMT* Met/Met and *OPRM1* AA ($p < 0.012$) on morphine dose. We also conducted multivariable linear regression analyses, with morphine dose as a continuous variable (analyses not shown). We observed statistical significance for the joint effects of *COMT* Met/Met and *OPRM1* AA ($p < 0.012$) on morphine dose.

4. Discussion

This study examined the potential joint effect of genes in predicting the clinical efficacy of morphine for cancer pain treatment and control. A number of studies have looked at the joint effects of genes in diseases like asthma (Hong et al., 2005), diabetes (Bergholdt et al., 2005; Maier et al., 2005), prostate (Xu et al., 2005) and lung cancer (Zhang et al., 2006), Alzheimer's disease (Infante et al., 2004), heart disease (Ye et al., 2003). The joint effects of genes can be expected to enhance, suppress or have no effect on the phenotypic outcome of interest.

Our findings provide empirical support for the importance of joint effects of the *OPRM1* and *COMT* gene in the clinical efficacy of morphine. We have shown that carriers of Met/Met and AA genotype in the *COMT* and *OPRM1* gene, respectively, needed less morphine dose for pain relief, thus providing preliminary support for the potential use of genetic data in predicting morphine dose for adequate control of pain in cancer patients. To our knowledge this is the first study to have looked at the joint effects of genes in opioid analgesia.

Recent debates on the assessment of candidate genes for pain and pain-related traits have focused on the need for a polygenic model for these complex phenotypes. Ideally, many genes with functional significance should be assessed. We selected the *OPRM1* and the *COMT* variants in this study because of the strength of previously published associations of these genes with pain and pain-related phenotypes and the minor allele frequencies (Hoehe et al., 2000; Mayer and Hollt, 2001).

Human studies showed the importance of the A118G polymorphism in pain and pain-related phenotypes. Fillingim and colleagues showed that the A118G polymorphism was associated with pressure pain sensitivity (Fillingim et al., 2005) and a recent study by Lotsch and Geisslinger (2006b) also showed that the A118G polymorphism is an important target for understanding variability in opioid efficacy as observed in human experimental pain models. We extended these findings by providing preliminary evidence of the effects of A118G polymorphism in the clinical efficacy of opioids and its joint effects with Val158Met.

That pain and pain-related phenotypes may also be modulated by the function of several endogenous substances such as adrenergic and noradrenergic neurotransmitters has also been shown in previous studies. Steiner and Gerfen (1998) found that the neuronal content of enkephalins is reduced by chronic activation of dopaminergic neurotransmission, which is followed by an up-regulation of mu-opioid receptor density in various regions of the brain (Chen et al., 1993; Steiner and Gerfen, 1998). Variable *COMT* enzyme activity may therefore alter dopaminergic activity and could, through an altered action of dopaminergic substances, have an influence on the enkephalin content and opioid receptor density.

Diatchenko and colleagues (2005) found haplotypes of the gene encoding *COMT* and found significant associations between the *COMT* haplotypes and pain sensitivity. Zubieta and colleagues found that Val158Met polymorphisms were associated with several pain phenotypes such as mu opioid system responses and higher sensory and affective ratings of pain (Zubieta et al., 2003). Homozygosity for the Met158 allele was associated with diminished regional mu-opioid system responses to pain and increases in μ-opioid receptor binding potential (Zubieta et al., 2003). The increase in the density of opioid receptors in those with Met/Met allele may therefore result in an improved efficacy of morphine. Rakvag et al. (2005) found that Val158Met polymorphism in the *COMT* gene is a significant predictor of morphine dose requirements for treatment of cancer pain. In this study, we found that *COMT* Val158Met had joint effects with the A118G polymorphisms in the *OPRM1* gene.

Table 2
Logistic regression model for morphine dose

Parameter	*p*-value	Odds ratio	95% Lower bound	95% Upper bound
Joint genotype groups*	0.05			
a. Met/Met & AA	0.012	0.278	0.102	0.756
b. AA but not Met/Met	0.280	0.625	0.266	1.467
c. Met/Met but not AA	0.191	0.240	0.028	2.039
Months using morphine	0.005	1.106	1.031	1.186
Time since cancer diagnosis (in months)	0.006	0.988	0.980	0.987

Morphine dose (low = ⩽117 mg/24 h; high > 117 mg/24 h).
* Reference variable is Neither Met/Met nor AA. Candidate variables included months using morphine, time since cancer diagnosis, age, sex, Karnofsky performance status, serum albumin, serum creatinine.

Klepstad et al. (2004) and Rakvag et al. (2005) assumed a gene-dose effect on univariate analyses, with carriers of *OPRM1* GG allele and *COMT* Val/Val allele associated with a higher morphine dose. However, we did not assume a gene-dose effect in the multivariate model, given the exploratory nature of this study. Future studies are needed to assess if there are gene-dose effects in the relationship between *OPRM1* and *COMT* genotypes and opioid efficacy.

We found that the duration of morphine treatment was an important predictor for morphine dose. It is possible that this time interval is reflective of the progression of disease, i.e., as patients progressed in their disease, they required higher morphine dose. Another explanation is that repeated administration of morphine leads to the need for higher dose or the need for opioid rotation. A known mechanism for this phenomenon is the reduction in the responsiveness of the G-protein coupled opioid receptors (Nestler, 1992) leading to either desensitization or downregulation. More recently the concept of paradoxical pain leading to analgesic tolerance has also been proposed (King et al., 2005).

There are limitations to this study. Arguably the design of our study may be associated with several biases, such as the heterogeneity of our study population. Another limitation is that the data were already previously analyzed for the individual effects of the OPRM1 and the COMT variant. Nonetheless the present analyses point to the importance of assessing the joint effects of genes on pain and pain-related phenotypes.

We also recognize that the complexity of morphine pharmacology suggests that the variability in opioid pain treatment is associated with genetic variation in several genes (Mogil, 1999; Mogil et al., 2000; Thompson et al., 2000; Flores and Mogil, 2001; Belfer et al., 2004; Duguay et al., 2004; Kim et al., 2004; Max, 2004; Diatchenko et al., 2005; Stamer et al., 2005; Lee et al., 2006; Lotsch and Geisslinger, 2006a).

Despite major improvement in pain control over the last 15 years, cancer-related pain continues to be a significant public health concern. Morphine is recommended as a first line strong opioid (World Health Organisation Geneva, 1996). The appropriate use and the ability to predict the optimal dose of opioids for cancer patients are crucial aspects for the effective treatment and management of cancer pain. Previous studies have focused on disease-related variables, clinical health status and sociodemographic characteristics in understanding adequate treatment and control of pain. Advances in molecular technology have now made it possible to assess the contribution of genes in pain treatment and control. Our observation that genetic differences influence clinical efficacy of morphine may prove useful in managing patients who receive these drugs, and importantly, preventing negative responses due to inappropriate dosing. Because pain is prevalent not just in cancer patients but in other diseases, the *COMT* and *OPRM1* genotypes may be relevant information to consider when implementing pain therapy.

In conclusion, our preliminary findings suggest the importance of assessing joint effects of genes in studies of clinical efficacy of morphine. Future studies with larger cohorts are needed to further characterize the joint effects of multiple genes, along with demographic and clinical variables, in predicting opioid dose.

Acknowledgement

The corresponding author is a recipient of CA109043-Career Development Grant from the National Institute of Health (NIH/NCI) and NIH Fellow, NINR Summer Genetics Institute, 2005.

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Paper IV

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Genetic variation in the *Catechol-O-Methyltransferase (COMT)* gene and morphine requirements in cancer patients with pain

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Published: 18 December 2008

Molecular Pain 2008, **4**:64 doi:10.1186/1744-8069-4-64

Received: 2 October 2008
Accepted: 18 December 2008

This article is available from: http://stephenslab.uchicago.edu/home.html

Abstract

Background: Genetic variation contributes to differences in pain sensitivity and response to different analgesics. Catecholamines are involved in the modulation of pain and are partly metabolized by the catechol-O-methyltransferase (COMT) enzyme. Genetic variability in the *COMT* gene may therefore contribute to differences in pain sensitivity and response to analgesics. It is shown that a polymorphism in the *COMT* gene, Rs4680 (Val158Met), influence pain sensitivity in human experimental pain and the efficacy for morphine in cancer pain treatment. In this study we wanted to investigate if variability in other regions in the *COMT* gene also contributes to interindividual variability in morphine efficacy.

Results: We genotyped 11 single nucleotide polymorphisms (SNPs) throughout the *COMT* gene, and constructed haplotypes from these 11 SNPs, which were in Hardy-Weinberg equilibrium. We compared both genotypes and haplotypes against pharmacological, demographical and patient symptoms measurements in a Caucasian cancer patient cohort (n = 197) receiving oral morphine treatment for cancer pain. There were two frequent haplotypes (34.5% and 17.8%) in our cohort. Multivariate analyses showed that patients carrying the most frequent haplotype (34.5%) needed lower morphine doses than patients not carrying the haplotype, with a reduction factor of 0.71 (p = 0.005). On the allele level, carriers of alleles for six of the SNPs show weak associations in respect to morphine dose and the alleles associated with the lowest morphine doses constitute part of the most frequent haplotype.

Conclusion: This study suggests that genetic variability in the *COMT* gene influence the efficacy of morphine in cancer patients with pain, and that increased understanding of this variability is reached by expanding from analyses of single SNPs to haplotype construction and analyses.

Background

One of the genes in which variability is believed to contribute to differences in pain sensitivity and response to analgesics is the *catechol-O-methyltransferase* (*COMT*) gene [1-3]. The COMT enzyme metabolises catecholamines such as dopamine, noradrenaline and adrenaline. The most studied single nucleotide polymorphism (SNP) in the *COMT* gene is the Rs4680, also known as Val158Met. This polymorphism causes a substitution from a valine (Val) to a methionine (Met) at amino acid position 158, leading to a three- to four-fold reduced activity of the COMT enzyme [4]. Because of the influence on COMT activity by the Rs4680 (Val158Met) SNP and the well established involvement of catecholamines in pain perception [5-7], several studies have investigated if this SNP can explain interindividual variability in pain perception and efficacy of analgesics. Zubieta *et al.*, demonstrated that individuals with the Met/Met genotype had higher sensory and affective ratings of pain and a higher regional density of mu opioid receptors in the brain [1]. The Rs4680 (Val158Met) SNP has also been shown to influence efficacy of morphine used for cancer pain, for which the Met/Met genotype group needed lower morphine doses than Val/Val genotype group [2]. Results from these two studies are intriguing since individuals with the Met/Met genotype report higher pain ratings, but need less morphine. However, as authors discuss [2], the increase of mu opioid receptor density seen in Met/Met genotype individuals [1], may explain why morphine is more effective in individuals carrying this genotype.

Other researchers have investigated other SNPs across the *COMT* gene and shown that other regions of the gene may also contribute to pain perception [3,8] and influence morphine-related side-effects [9]. Diatchenko *et al.*, identified three genetic variants (haplotypes) in the *COMT* gene and designated them as low pain sensitivity (LPS), average pain sensitivity (APS) and high pain sensitivity (HPS) haplotypes. The Rs4680 (Val158Met) polymorphism was one of four SNPs included in their haplotype analyses. The authors argue that the Rs4680 (Val158Met) SNP cannot account for the observed variations in pain perception alone, since both the LPS and HPS haplotypes possess the G allele that codes for the more stable Val variant of the COMT enzyme [3]. Kim *et al.*, analysed 13 SNPs in the *COMT* gene and their association to acute post-surgical pain in humans [8]. The authors found that the Rs740603 polymorphism showed significant association with maximum post-operative pain rating, but did not observe any association between other SNPs, including the Rs4680 (Val158Met) SNP, and pain score. Ross *et al.*, found that a SNP in intron 1 (Rs740603) and a haplotype, defined by SNPs in the promoter region and intron 1, were significantly associated with drowsiness and confusion or hallucinations in a cancer patient cohort treated with morphine. In the study by Ross *et al.*, the Rs4680 (Val158Met) SNP did not influence the risk for morphine induced adverse effect [9].

All the three studies cited above that have investigated multiple SNPs in the *COMT* gene have either pain perception or the risk for opioid adverse effects as the primary endpoint in the study [3,8,9]. No studies have investigated if other SNPs than the Rs4680 (Val158Met) in the *COMT* gene are important for the analgesic efficacy of morphine. Therefore, in a patient cohort in which we have previously shown that the Rs4680 (Val158Met) polymorphism influences the efficacy of morphine for cancer pain [2], we investigated if variability in other regions in the *COMT* gene also contribute to interindividual variability in morphine efficacy. In addition to examining the effect from each individual SNP we constructed long haplotypes in order to study composite effect from combinations of 11 SNPs along the gene.

Results

DNA from 197 patients receiving oral morphine treatment for cancer pain was analysed in this study.

Genotype and haplotype distribution

A schematic presentation of the 11 SNPs analysed in the *COMT* gene is shown in Figure 1. The genotype frequencies, allele frequencies and allele carriage for all 11 SNPs analysed are shown in Table 1. All SNPs were in Hardy-Weinberg equilibrium. The long haplotypes constructed from the 11 SNPs in the *COMT* gene are shown in Table 2. The frequencies of the two most common haplotypes were 34.5% and 17.8%. Fourteen different haplotypes with a frequency of > 1% described 91% of the population. We designated the haplotypes as haplotype 1 to haplotype 14, corresponding to the frequency at which they occur; haplotype 1 being the most frequent.

Morphine dose and genotypes

The pharmacological observations for genotype groups and allele carriage are shown in Table 3. The median morphine dose requirements between genotype groups for the Rs4818 polymorphism were 60, 80 and 120 mg/24 h for the CC, CG and GG genotype groups, respectively ($p = 0.042$) and for the Rs4680 (Val158Met) polymorphism the median morphine doses were 90, 80 and 60 mg/24 h for the GG, GA and AA genotype groups, respectively ($p = 0.022$). For six of the SNPs (Rs5746849, Rs740603 in intron 1, Rs6269 in intron 2, Rs2239393 in intron 3 and Rs4818 and Rs4680 (Val158Met) in exon 4) allele carriers showed a tendency to differences in median morphine doses. (Table 3).

Table 1: *Catechol-O-methyltransferase* (*COMT*) genotype frequencies, allele frequencies and allele carriage in the total of 197 cancer patients

SNP (region)	Genotype	Genotype frequencies	Allele	Allele frequencies	Allele carriage
Rs2075507* (promoter)	AA	0.26	A	0.53	0.80
	AG	0.54	G	0.47	0.74
	GG	0.20			
Rs737866 (intron 1)	AA	0.61	A	0.78	0.94
	AG	0.33	G	0.22	0.39
	GG	0.06			
Rs7287550 (intron 1)	CC	0.53	C	0.72	0.91
	CT	0.38	T	0.28	0.47
	TT	0.09			
Rs5746849 (intron 1)	GG	0.18	G	0.43	0.68
	GA	0.49	A	0.57	0.82
	AA	0.33			
Rs740603 (intron 1)	AA	0.31	A	0.56	0.81
	AG	0.50	G	0.44	0.69
	GG	0.19			
Rs6269 (intron 2)	AA	0.40	A	0.62	0.84
	AG	0.44	G	0.38	0.60
	GG	0.16			
Rs2239393 (intron 3)	AA	0.40	A	0.62	0.84
	AG	0.44	G	0.38	0.59
	GG	0.16			
Rs4818 (exon 4)	CC	0.41	C	0.63	0.84
	CG	0.43	G	0.37	0.59
	GG	0.16			
Rs4680 (Val158Met) (exon 4)	GG	0.22	G	0.44	0.66
	GA	0.44	A	0.56	0.78
	AA	0.34			
Rs174699 (intron 5)	CT	0.09	C	0.04	0.09
	TT	0.91	T	0.96	100.0
Rs165728 (untranslated region)	CT	0.10	C	0.05	0.10
	TT	0.90	T	0.95	100.0

* Rs2075507 has recently been revised, earlier SNP number was Rs2097603

Morphine dose and haplotypes

We observed that carriers of haplotype 1, the most frequent haplotype in this Caucasian population (Table 2), needed less morphine than non-carriers, with a median morphine dose of 60 mg/24 h for carriers versus 100 mg/24 h for non-carriers (p = 0.006) (Table 4a). The serum concentrations of morphine, morphine-6-glucuoride (M6G) and morphine-3-glucuronide (M3G) reflected the different morphine doses between haplotypes, but no differences were statistically significant (Table 4b).

Patient symptoms including average pain, fatigue, nausea and vomiting, dyspnea, sleep, appetite, constipation and cognitive function were similar for carriers and non-carriers of haplotype 1 (Table 5). Also the patient characteristics age, gender, tumour diagnosis, performance status, creatinine and albumin serum concentration, time since morphine treatment started and survival time after study were similar between the two genetic groups (Table 6). We observed that the carriers of haplotype 1 have had the cancer diagnosis longer (45 months) than non-carriers of

Table 2: COMT haplotype frequencies.

	SNP position (5' to 3')											Haplotype frequency
Haplotype	Rs 2075507	Rs 737866	Rs 7287550	Rs 5746849	Rs 740603	Rs 6269	Rs 2239393	Rs 4818	Rs 4680 (Val158Met)	Rs 174699	Rs 165728	n chromosomes (%)
1	G	A	C	A	A	A	A	C	A	T	T	136 (34.5)
2	A	G	C	G	G	G	G	G	G	T	T	70 (17.8)
3	A	A	T	G	G	A	A	C	A	T	T	33 (8.4)
4	A	A	T	G	G	G	G	G	G	T	T	24 (6.1)
5	G	A	C	A	A	G	G	G	G	T	T	18 (4.6)
6	A	A	T	A	A	A	A	C	A	T	T	18 (4.6)
7	G	A	C	G	G	G	G	G	G	T	T	10 (2.5)
8	A	A	C	A	A	G	G	G	G	T	T	9 (2.3)
9	A	A	T	G	G	A	A	C	G	T	T	9 (2.3)
10	A	A	C	A	A	A	A	C	A	T	T	9 (2.3)
11	A	A	T	A	A	A	A	C	G	C	C	8 (2.0)
12	A	A	T	A	G	A	A	C	A	T	T	6 (1.5)
13	G	A	T	A	A	G	G	C	G	T	T	4 (1.0)
14	G	G	C	A	A	A	A	C	A	T	T	4 (1.0)
X	-	-	-	-	-	-	-	-	-	-	-	36 (9.1)

Catechol-O-methyltransferase (*COMT*) haplotype frequencies for the total of 197 cancer patients.

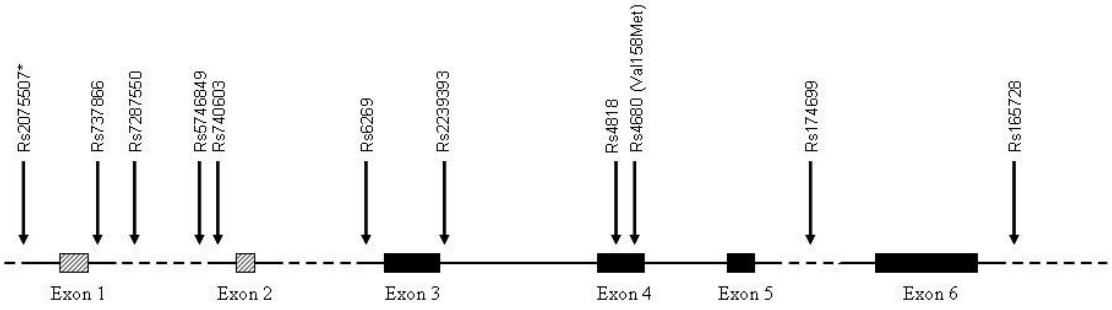

Figure 1
Schematic diagram of the *COMT* gene. Schematic diagram of the *catechol-O-methyltransferase* (*COMT*) gene, labeled with the 11 SNPs analysed in the present study. *Rs2075507 has recently been revised, the former SNP number was Rs2097603. Exon 1 and exon 2 are non-coding.

haplotype 1 (31 months) (p = 0.03; Table 6). However there were no differences in time since morphine treatment started between carriers and non-carriers of haplotype 1 (3.4 and 3.6 months respectively; p = 0.47).

In a multivariate stepwise linear regression analysis the variables "time since morphine treatment started" and haplotype 1 were shown to influence the morphine dose (p = 0.001 and p = 0.005; Table 7). After adjusting for the variable "time since morphine treatment started", the carriers of haplotype 1 still require lower morphine doses than patients that do not carry haplotype 1. Time since morphine treatment started is positively associated to morphine dose, whereas the carriers of haplotype 1 is predicted to need lower doses of morphine than non-carriers of haplotype 1 with a reduction factor of 0.71 (see discussion for calculation).

Discussion

We have identified a frequent haplotype (haplotype 1) in the *COMT* gene that may influence the morphine dose requirements in cancer patients with pain. Patients who carry haplotype 1 need lower morphine doses to relieve pain than patients that do not carry this haplotype (p = 0.006). The carriers of haplotype 1 are also carriers of the A allele for the Rs4680 (Val158Met) polymorphism, which is in agreement with our earlier observation that carriers of the Met variant of the enzyme (= A allele) need lower morphine doses than carriers of the Val variant of the COMT enzyme [2]. However, the effect of the A allele for the Rs4680 (Val158Met) polymorphism is not seen for haplotype 3 (Table 4a).

The Rs4680 (Val158Met) polymorphism is the most studied SNP in the *COMT* gene because the valine (Val) to methionine (Met) substitution leads to a three-to four-fold reduced activity of the COMT enzyme [4], hence the Val/Val, Val/Met and Met/Met genotypes predict a high, intermediate and low COMT enzyme activity, respectively. As the COMT enzyme metabolises catecholamines, a low COMT enzyme activity could result in an enhanced activation of dopaminergic neurotransmission. It is shown in animal models that the neuronal content of enkephalin peptides is reduced by chronic activation of dopaminerg neurotransmission [10]. Pain sensitivity is affected by the neuronal content of enkephalin, and reduction in the enkephalin content is shown to be followed by an upregulation of mu opioid receptors [11]. Taken together, this can explain the influence from variation in the *COMT* gene on the effect of opioids in pain treatment.

We also observed that carriers of alleles for six of the SNPs analysed, the Rs5746849 and Rs740603 polymorphism in intron 1, the Rs6269 polymorphism in intron 2, the Rs2239393 polymorphism in intron 3 and the Rs4818 and Rs4680 (Val158Met) polymorphisms in exon 4 were weakly associated to morphine dose (Table 3). The alleles associated with the lowest morphine dose requirements constitute part of the SNP sequence in haplotype 1, which seems reasonable since haplotype 1 is associated with lower morphine dose requirements in this patient cohort. The SNPs defining a haplotype may have functional effects on a protein if the amino acid code is changed [4], and synonymous SNPs may have effects on the secondary structure of mRNA [12], that could alter mRNA stability and/or the translation of a protein [13]. SNPs may also be associated to a phenotype without having any effects neither on the protein nor the mRNA, if it is closely linked to another SNP exerting the real effect on the protein or

Table 3: Pharmacological observations.

SNP	Genotype	Morphine dose mg/24 h median [range]	P value	Allele carriage	Morphine dose mg/24 h Median [range]	P value
Rs2075507	AA	90 [20–420]	0.220	A	80 [20–760]	0.90
	AG	70 [20-480]		Not A	60 [10–660]	
	GG	60 [10–660]		G	70 [10–760]	0.09
				Not G	90 [20–420]	
Rs737866	AA	70 [10–660]	0.486	A	73 [10–760]	0.26
	AG	80 [20–760]		Not A	90 [20–350]	
	GG	90 [20–350]		G	80 [20–760]	0.45
				Not G	70 [10–660]	
Rs7287550	CC	70 [20–760]	0.862	C	78 [10–760]	0.59
	CT	80 [10–410]		Not C	120 [30–420]	
	TT	120 [30–420]		T	80 [10–420]	0.91
				Not T	70 [20–760]	
Rs5746849	GG	115 [20–420]	0.103	G	80 [20–420]	0.57
	GA	80 [20–390]		Not G	65 [10–760]	
	AA	65 [10–760]		A	70 [10–760]	0.03[c]
				Not A	115 [20–420]	
Rs740603	AA	70 [10–760]	0.099	A	70 [10–760]	0.04[c]
	AG	80 [20–390]		Not A	110 [20–420]	
	GG	110 [20–420]		G	80 [20–420]	0.92
				Not G	70 [10–760]	
Rs6269	AA	70 [20–660]	0.090	A	70 [10–660]	0.03[c]
	AG	75 [10–480]		Not A	120 [20–760]	
	GG	120 [20–760]		G	80 [10–760]	0.32
				Not G	70 [20–660]	
Rs2239393	AA	70 [20–660]	0.093	A	70 [10–660]	0.03[c]
	AG	73 [10–480]		Not A	120 [20–760]	
	GG	120 [20–760]		G	80 [10–760]	0.42
				Not G	70 [20–660]	
Rs4818	CC	60 [10–660]	0.042 [a]	C	70 [10–660]	0.04[c]
	CG	80 [20–480]		Not C	120 [20–760]	
	GG	120 [20–760]		G	80 [20–760]	0.04[c]
				Not G	60 [10–660]	
Rs4680 (Val158Met)	GG	90 [20–760]	0.022 [b]	G	80 [10–760]	0.045[c]
	GA	80 [10–480]		Not G	60 [20–660]	
	AA	60 [20–660]		A	70 [10–660]	0.07
				Not A	90 [20–760]	
Rs174699	CT	80 [20–480]	0.666	C	80 [20–480]	0.67
				Not C	73 [10–760]	
	TT	73 [10–760]		T	80 [10–760]	-
				Not T	-	
Rs165728	CT	80 [20–480]	0.457	C	80 [20–480]	0.46
				Not C	70 [10–760]	
	TT	70 [10–760]		T	80 [10–760]	-
				Not T	-	

a Kruskal-Wallis test for 3 independent samples; b Jonckheere Tepstra test for 3 independent samples; c Mann-Whitney U test for 2 independent samples.
Pharmacological observations for genotype groups and allele carriage.

Table 4: Pharmacological observations I % II

a – Pharmacological observations I.
Morphine dose and haplotype groups

Haplotype	Carriage	N	Morphine dose *mg/24 h* median [range]	P value
1	Yes	114	60 [10–660]	0.006 [a]
	No	83	100 [20–760]	
2	Yes	61	80 [20–410]	0.94
	No	136	70 [10–760]	
3	Yes	31	90 [20–420]	0.44
	No	166	78 [10–760]	
4	Yes	24	70 [20–420]	0.94
	No	173	80 [10–760]	
5	Yes	17	120 [20–760]	0.17
	No	180	78 [10–660]	
6	Yes	17	120 [30–200]	0.56
	No	180	73 [10–760]	
7	Yes	10	120 [40–290]	0.44
	No	187	80 [10–760]	

N = number of patients
a Mann-Whitney U test for independent samples.

b – Pharmacological observations II.
Serum concentration of morphine, M6G and M3G against haplotype groups

Haplotype	Carriage	N	Morphine (*nmol/ml*) median [range]	M6G (*nmol/ml*) median [range]	M3G (*nmol/ml*) median [range]
1	Yes	114	51 [2–350]	310 [10–2660]	1810 [120–16200]
	No	83	59 [3–1070]	349 [20–4830]	2310 [110–21250]
2	Yes	61	50 [3–330]	346 [20–2482]	1890 [120–12390]
	No	136	60 [2–1070]	319 [10–4830]	2040 [110–21250]
3	Yes	31	67 [5–320]	310 [29–1690]	2200 [197–7780]
	No	166	50 [2–1070]	330 [10–4830]	1992 [110–21250]
4	Yes	24	51 [3–277]	380 [20–2482]	2213 [110–12390]
	No	173	57 [2–1070]	320 [10–4830]	1992 [120–21250]
5	Yes	17	80 [4–1070]	403 [20–4830]	2415 [110–21250]
	No	180	52 [2–519]	325 [10–2660]	1960 [120–16200]
6	Yes	17	90 [9–230]	470 [120–1105]	3118 [1017–5481]
	No	180	50 [2–1070]	318 [10–4830]	1890 [110–21250]
7	Yes	10	50 [6–220]	460 [81–1809]	1832 [490–9460]
	No	187	58 [2–1070]	330 [10–4830]	2020 [110–21250]

N = number of patients; M6G = Morphine-6-glucuronide; M3G = Morphine-3-glucuronide
No statistical differences between carriers and non-carriers of the different haplotype groups for morphine, M6G and M3G serum concentration

Table 5: Patient symptoms.

	Haplotype 1		P value [a]
	Carriers	Non-carriers	
BPI average pain	3.5 (2.6)	3.9 (2.2)	0.26
Fatigue (EORTC score)	64.5 (23.5)	68.6 (23.1)	0.28
Nausea and vomiting (EORTC score)	26.6 (25.9)	27.0 (28.5)	0.77
Dyspnea (EORTC score)	36.6 (32.5)	34.4 (34.7)	0.80
Sleep (EORTC score)	35.3 (36.0)	32.8 (35.2)	0.58
Appetite (EORTC score)	53.2 (37.6)	54.3 (37.2)	0.95
Constipation (EORTC score)	54.5 (37.9)	55.7 (38.4)	0.77
Mini mental examination sum score	26.1 (3.4)	25.6 (4.0)	0.66

a Mann-Whitney U test for 2 independent samples
Patient symptoms for carriers and non-carriers of haplotype 1.

mRNA. The exact contribution from each SNP in haplotype 1 to the observed effect on morphine requirements in the present study is not known.

In the paper we have constructed long haplotypes across the entire *COMT* gene. An alternative approach would have been to construct haplotypes defined by haploblock boundaries. The latter approach is based on including only SNPs that have a very high probability of being inherited together (visualized by the value of D' or r^2 which are correlation factors between SNPs) and as a consequence limiting the gene distance to which SNPs categorize into haplotypes. According to literature the *COMT* gene consists of at least three haploblocks in Caucasians [3,14] and there is consistency between ethnic groups, so the haploblocks is likely to be present also in a Norwegian population. The division of genes into haploblocks limits the number of haplotypes present in the population and thereby increases the number of individuals that fall into each different haplotype group. When analysing long haplotypes across the entire gene fewer individuals in the population will be carriers, but more information will be gained from the effect of combination of SNPs and in that sense long haplotypes may be more biologically relevant. Any sizes of haplotypes will be of more scientifically interests than analyses of SNPs considered one by one.

A cancer population is a heterogeneous group and prone to be influenced from several possible confounders such as severity of disease, organ dysfunction and treatment of other drugs. Therefore, we analysed for possible confounding factors that could influence the need for morphine in cancer pain. We found no differences between carriers and non-carriers of haplotype 1 for patients'

Table 6: Patient demographics.

	Haplotype 1		P value
	Carriers	Non-carriers	
Age	63 (13)	64 (12)	0.68
Gender:			
Male	68 (60%)	44 (53%)	0.38
Female	46 (40%)	39 (47%)	
Tumour diagnosis:			
Urological	38	19	0.80
Lung	20	17	
Breast	25	19	
Gastrointestinal	7	10	
Haematological	10	6	
Others	14	12	
Karnofsky performance status	67 (14)	66 (13)	0.36
Creatinine serum (μmol/l)	86 (28)	87 (39)	0.48
Albumin serum (g/l)	33 (5)	32 (5)	0.12
Time since diagnosis (months)	45 (52)	31 (43)	0.03[a]
Time since morphine treatment started (months)	3.4 (7.8)	3.6 (5.9)	0.47
Survival time after study (months)	5.7 (6.2)	4.8 (5.5)	0.23

Numbers in the table are given as mean (SD) or absolute numbers (%)
a Mann-Whitney U test for 2 independent samples
Patient demographics for carriers and non-carriers of haplotype 1.

Table 7: Regression analysis. Morphine dose regression analysis

	b	SE	P value
Haplotype 1	-0.147	0.051	0.005
Time since morphine treatment started	0.013	0.004	0.001
Constant	1.95	0.042	

The logarithm (log_{10}) of the 24 hour oral morphine dose was the dependent variable in this regression analysis. The regression coefficient, b, is an estimate of the parameter beta. SE = standard errors

symptoms or for patients' demographics, except from the time since diagnosis. There was a tendency that carriers of haplotype 1 have had a cancer diagnosis for a longer time than non-carriers of the haplotype (Table 6). Theoretically, patients with a diagnosis for a long time (that is the patients carrying the haplotype 1) should need more morphine due to more advance cancer disease. In our cohort the carriers of haplotype 1 need less morphine than non-carriers. Thus, a potential bias from the skewed distribution of time since cancer diagnosis is that the observed difference between haplotypes is lower that the true difference between haplotypes. However, in order to further explore if time since diagnosis was an independent predictor of morphine dose we included potential confounding factors in a multivariate analysis. This analysis showed that only "haplotype 1" and "time since morphine treatment started" were predictors for morphine dose. Regression analysis is usually linear, where b is the slope of the graph and gives the change in value of one outcome (e.g. morphine dose), per unit change in the other (e.g. months of morphine treatment). In our regression the association is not linear because we used the logarithm (log_{10}) of the 24 hour morphine dose as the dependent variable. Therefore, for each month of morphine treatment, the predicted 24 hour morphine dose increases by a factor of $10^{(b \times months)}$ which translates to that the dose on average increases by 43% every 12 months ($10^{0.013 \times 12}$). Patient carrying haplotype 1 is predicted to need less morphine to relieve pain than a patient not carrying haplotype 1, with a reduction factor of $10^{(b)} = 10^{(-0.147)} = 0.713$. In other words, if a patient, not carrying haplotype 1 need 100 mg of morphine to relieve pain, a patient carrying haplotype 1 is predicted to need 71 mg of morphine to relieve similar pain. The difference we observe in the median morphine dose between non-carriers and carriers of haplotype 1 is of similar order of magnitude, 100 mg versus 60 mg of the 24 hour morphine dose respectively (Table 4a). Experimental studies including healthy volunteers give more controlled experimental conditions due to less potential confounders. However, clinical studies including cancer patients, such as this study and the study by Ross *et al.*,[9] are needed to observe if genetic variability do influence morphine treatment in the patients actually receiving the drug. The best effort in a clinical population is therefore to include potential confounders in the analyses and interpret findings within the clinical context.

Ross *et al.*, analysed the *COMT* gene and its association with the central side effects of morphine in a cancer patient cohort. They found that a haplotype present in 10.4% of the population was associated to drowsiness and confusion or hallucination [9]. SNPs in the promoter region and in the intron 1 region define this haplotype and the authors suggest that it is this region of the *COMT* gene that is of interest in order to explain clinical effect from the COMT enzyme. Alterations in the promoter and intronic region of the gene can influence the regulation of gene expression. Therefore, polymorphisms in these regions might be as important as functional SNPs in coding regions. The Ross study did not find any associations between the Rs4680 (Val158Met) polymorphism and central side effects of morphine [9]. Haplotype 1 in the present study is not identical to the haplotype that Ross and co-authors observed to be associated to central side effects of morphine. However, the haplotypes identified as important by Ross *et al.*, and haplotype 1 in our study are related as 7 of 10 possible SNP positions from the Rs5746849 polymorphism in intron 1 to the UTR' region carry the same allele and both haplotypes carry the A allele at the Rs4680 (Val158Met) polymorphism. An explanation for the discrepancy of the haplotypes might be that efficacy for pain relief and risks of adverse effects have different relationships to genotypes.

The need for morphine is a result of both the efficacy of morphine and influenced by the patients' pain perception. Patients can experience variable pain from a given nociceptive stimuli. Therefore genetic variability related to opioid efficacy as studied in the present study is closely linked to genetic variability related to pain perception.

Diatchenko *et al.*, have investigated *COMT* gene variability and association to pain responses [3]. They identified three haplotypes in the *COMT* gene strongly associated to pain sensitivity and they designated the different haplotypes as low pain sensitivity (LPS), average pain sensitivity (APS) and high pain sensitivity (HPS) haplotypes. Four SNPs (Rs6269, Rs4633, Rs4818 and Rs4680) constitute these haplotypes, of which three of the SNPs (Rs6269, Rs4818 and Rs4680) are included in our analyses. However, Diatchenko *et al.*, did not include the region in intron 1 or the promoter regions, the region which the study by Ross and co-authors [9] believe to be the functional region of interest in the *COMT* gene. A direct comparison with our study is difficult because we have included 11 SNPs in our haplotype analyses while Diatchenko *et al.*, focused on four SNPs. Kim *et al.*, have also

investigated *COMT* gene variability and association to pain responses and found that the Rs740603 SNP was associated with maximum post-operative ratings of pain. Even though the comparison between Diatchenko *et al.*, [3] and Kim *et al.*, [8] with our findings is important, it is also complicated because we investigate the morphine efficacy while they are studying the genetics of pain sensitivity. However, one agreement between the different studies is that the Rs4680 (Val158Met) polymorphism is not the sole explanation of why COMT seem to contribute to the effect on pain perception or opioid efficacy as first reported by Zubieta *et al.*, [1] and Rakvag *et al.*, [2], respectively.

In the present study the serum concentrations of morphine, morphine-6-glucuoride (M6G) and morphine-3-glucuronide (M3G) reflected the different morphine doses between haplotypes (Table 4b), but did not reach statistical significance as seen for the morphine dose. The interindividual variation of serum concentrations is more pronounced than for the morphine doses. Consequently, a larger effect size or an increased number of patients are needed to reach a statistical significance for an observed difference between serum concentrations.

Numerous SNPs have been detected in the *COMT* gene and 22 of the most frequent SNPs have been analysed regarding different aspects of pain and opioid responses [2,3,8,9], so the analyses of 11 SNPs in our study do not cover all genetic variation in the *COMT* gene. However, as many SNPs are tightly linked within haploblocks, most genetic variability is captured if the selections of SNPs are chosen to represent the different haploblocks building the entire gene [15], which is done in the present study.

In the present study we have carried out several comparisons. Multiple test correction, as the Bonferroni, is used when tests are independent and is therefore highly conservative. In a genetic association study where SNPs usually are partly linked to each other, as is the case for the *COMT* gene, a conservative multiple test correction lead to missing real differences [16,17]. Also, in our study the haplotype analyses is the primary outcome and then as a consequence we do not consider all null hypotheses to be of equally importance [17]. In addition to the haplotype analyses, differences at genotype level and allele level are presented in this study, but differences at $0.01 < p < 0.05$ are interpreted with caution and reported as weak associations between genetic groups.

Conclusion

This study suggest that genetic variability in the *COMT* gene influence the efficacy of morphine in cancer pain patients, and that increased understanding of this variability is reached by expanding from analyses of single SNPs to haplotype constructions and analyses.

Materials and methods

Ethics

The study was carried out in accordance to the principles of the Helsinki declaration. The Regional Committee for Medical Research Ethics, Health Region IV, Norway, approved the study. All patients gave their oral and written informed consent before inclusion in the study.

Subjects

We investigated the same cohort as previously described by Rakvag *et al.*, [2]. Two hundred and seven patients were included in the original study, but blood for further genetic analyses was not available for 10 patients. Therefore, 197 patients were available for further genotyping and included in our analyses. All 197 patients were Caucasians, and all received scheduled oral morphine for cancer pain treatment.

Assessments

Pain was measured using the item "average pain" during the last 24 hours in the Brief Pain Inventory (BPI) questionnaire. The patients rated pain on an 11-point numeric scale, where 0 represents "no pain" and 10 represents "pain as bad as you can imagine". The BPI is developed for the use in cancer pain patients, validated in Norwegian, and recommended by the European Association of Palliative Care for use in clinical studies [18-20]. The European Organization for Research and Treatment of Cancer core quality-of-life questionnaire (EORTC QLQ-C30) version 3.0 was used to assess the patients' nausea/vomiting, constipation, fatigue, sleep, appetite and dyspnea [21]. Cognitive function was assessed with the Mini Mental State (MMS) examination. The MMS score ranges from 0 to 30, higher scores meaning better cognitive function [22]. The patients' functional status was assessed by the Karnofsky performance status [23]. Survival time, time since start of morphine, cancer diagnoses and opioid doses were obtained from the patients' hospital records.

Blood samples and pharmacogenetic analyses

Collection of blood samples and determination of serum concentration of morphine and its metabolites (morphine-6-glucuronide and morphine-3-glucuronide) were done as described in a previous work from our group [24]. Creatinine serum concentrations and albumin serum concentrations were measured using standard analytical methods.

The genotyping was performed at the Clinical Genomics Group, Imperial College in London, UK. The selection of SNPs for this study and primer sequences for sequence specific polymerase chain reaction (SSP-PCR) are

described in a study by Ross and co-authors investigating another cohort and another primary outcome [9]. The selection was based upon frequency of SNP, position in gene and what was known in the literature at the time research was planned. Of the 13 polymorphic SNPs included in Ross and co-authors' study, the rs174680 and the rs7290221 polymorphisms in intron 1, were not analysed in our patient cohort due to very tight linkage with the rs7287550 polymorphism and the rs5746849 polymorphism respectively. As the reaction of the Rs4633 polymorphism did not work very well, we excluded this polymorphism in the present study, but included the Rs4818 polymorphism in exon 4, which had not been analysed in the previous Ross study. Together, 11 SNPs were genotyped in the present study.

Genomic DNA was isolated from 50 to 200 μL EDTA blood on a MagNA Pure LC (Roche Diagnostics Scandinavia AB, Bromma, Sweden) using the MagNA Pure LC DNA Isolation Kit I applying the manufacturers high performance protocol. Purified genomic DNA was eluted in 100 μL antiseptic water and stored at -20°C. Genotypes were determined using sequence specific primers in a polymerase chain reaction (SSP-PCR) [25]. A sequence specific primer and a consensus primer produce a DNA product of known size in this PCR. The sequence specific primer has a mismatch at the 3' end which is designed to identify each genotype variant. The PCR were carried out as described in Ross et al. [26]. PCR products were then electrophoresed on 1.5% agarose gels (Bioline Ltd, London, UK) containing 0.14 mg/mL ethidium bromide (Sigma Ltd, Poole, UK), at 200 volts/cm^2 in 0.5% tris borate EDTA buffer (Sigma Ltd, Poole, UK). Products were visualised with a UV illuminator and photographed with a Polaroid camera. The presence of an allele specific band of the expected size, in conjunction with a control band was used to identify an allele.

Construction of haplotypes

Genotype and allele frequencies and allele carriage were calculated and checked for Hardy-Weinberg equilibrium. Haplotypes were constructed from genotype information from each patient using the computer program Phase http://stephenslab.uchicago.edu/home.html[27,28].

Statistical analyses

The statistical software SPSS for windows v. 14.0 was used to run non-parametric tests and to run a stepwise linear regression analysis. Because the *COMT* alleles are expected to be codominant with respect to the Rs4680 (Val158Met) polymorphism and COMT enzyme activity, we used the Jonckheere-Terpstra test for comparison between genotype groups, working with a hypothesis that $\mu_1 \leq \mu_2 \leq \mu_3$ (or the opposite $\mu_1 \geq \mu_2 \geq \mu_3$) [29]. For all other SNPs we used the Kruskal-Wallis test for comparison between genotype groups. We used the logarithm ($\log_{10}$) of the 24 hour morphine dose as the dependent variable in the regression analyses because the 24 hour morphine dose, as expected, did not display a normal distribution. The analysis was a stepwise enter linear regression with a criterion for removal of a variable of $p > 0.1$. The variables included in the regression analysis as independent variables were: haplotype 1, age, gender, tumour diagnosis, Karnofsky performance status, creatinine and albumin serum concentration, time since diagnosis, time since morphine treatment started, survival time after study, BPI average pain score, EORTC score for fatigue, nausea and vomiting, dyspnea, sleep, appetite and constipation, and finally the sum score for the Mini mental examination measuring cognitive function.

Interpretation of p values in this study is done with caution considering the multiplicity of tests carried out.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PK, SK, FS and TTR conceived of the study and PK, FS and SK collected the experimental data. TTR, JRR and HS carried out the molecular genetics and statistical analysis. All authors drafted the manuscript and approved the final version.

Acknowledgements

We wish to thank the members of the "Pain and Palliation Research Group" at NTNU, Trondheim, and the members, especially Sophy Gretton, of the "Clinical Genomic Group" at the Imperial College, London, for valuable discussion and support. We also want to thank Peter Fayers for discussions on the statistical methods applied in this paper. And finally, we want to thank Fredrik Ekeberg Johansen for contribution on the haplotype analyses. This work was supported by grants from The Research Council of Norway, Norwegian University of Science and Technology, and the Central Norway Health Authority.

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Appendix

I. Brief Pain Inventory
II. EORTC QLQ-C30
III. Minimental status (MMS)
IV. Karnofsky Performance Scale

NTNU DMF, IKM

Brief Pain Inventory

New version

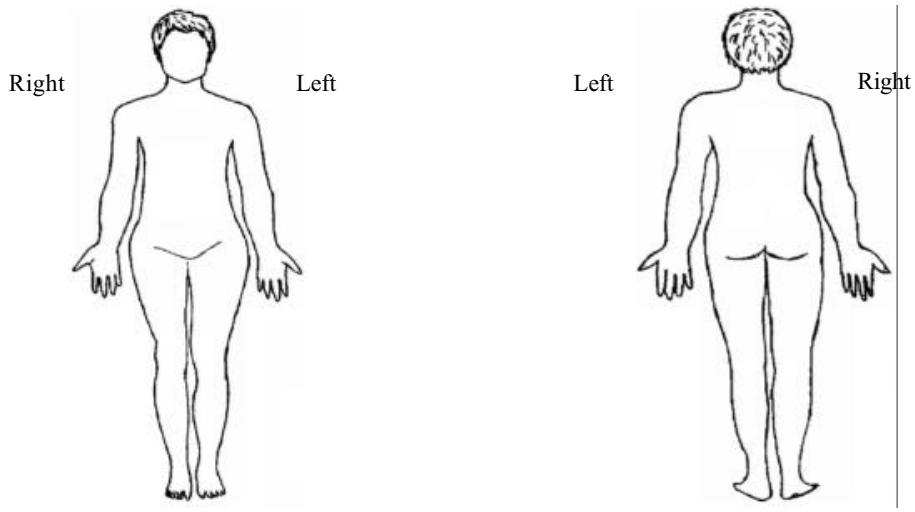
EAPC ONLUS

Projectno.:
OPI 03-006

1. Throughout our lives, most of us have had pain from time to time (such as minor headaches, sprains, and toothaches). Have you had pain other than these everyday kinds of pain today?

   ☐ Yes ☐ No

2. On the diagram, shade in the areas where you feel pain. Put an X on the area that hurts the most.



3. Please rate your pain by circling the one number that best describes your pain at its worst in the last 24 hours.

0 1 2 3 4 5 6 7 8 9 10

No pain — Pain as bad as you can imagine

4. Please rate your pain by circling the one number that best describes your pain at its least in the last 24 hours.

0 1 2 3 4 5 6 7 8 9 10

No pain — Pain as bad as you can imagine

5. Please rate your pain by circling the one number that best describes your pain on the average.

0 1 2 3 4 5 6 7 8 9 10

No pain — Pain as bad as you can imagine

6. Please rate your pain by circling the one number that tells how much pain you have right now.

0 1 2 3 4 5 6 7 8 9 10

No pain — Pain as bad as you can imagine

CRF no: | | | | |

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Please go to the next page

7. In the last 24 hours, how much relief have pain treatments or medications provided? Please circle the one percentage that most shows how much relief you have recieved.

0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%

No Relief **Complete Relief**

Circle the one number that describes how, during the past 24 hours, pain has interfered with your:

8. General Activity

0 1 2 3 4 5 6 7 8 9 10

Does not Interfere Completely Interferes

9. Mood

0 1 2 3 4 5 6 7 8 9 10

Does not Interfere Completely Interferes

10. Walking Ability

0 1 2 3 4 5 6 7 8 9 10

Does not Interfere Completely Interferes

11. Normal work (includes both work outside the home and housework)

0 1 2 3 4 5 6 7 8 9 10

Does not Interfere Completely Interferes

12. Relations with other people

0 1 2 3 4 5 6 7 8 9 10

Does not Interfere Completely Interferes

13. Sleep

0 1 2 3 4 5 6 7 8 9 10

Does not Interfere Completely Interferes

14. Enjoyment of life

0 1 2 3 4 5 6 7 8 9 10

Does not Interfere Completely Interferes

NTNU DMF, IKM

EORTC QLQ-C30

(version 3)

EAPC ONLUS

Projectno.:
OPI 03-006

We are interested in some things about you and your health. Please answer all of these questions yourself by ticking the alternative that best applies to you. There are no "right" or "wrong" answers. The information that you provide will remain strictly confidential.

	Not at all	A little	Quite a bit	Very much
1. Do tou have any trouble doing strenuous activities, like carrying a heavy shopping bag or a suitcase?	☐	☐	☐	☐
2. Do you have any trouble taking a long walk?	☐	☐	☐	☐
3. Do you have any trouble taking a short walk outside of the house?	☐	☐	☐	☐
4. Do you need to stay in bed or a chair during the day?	☐	☐	☐	☐
5. Do you need help with eating, dressing, washing yourself or using the toilet?	☐	☐	☐	☐

During the past week:	Not at all	A little	Quite a bit	Very much
6. Were you limited in doing either your work or other daily activities?	☐	☐	☐	☐
7. Were you limited in pursuing your hobbies or other leisure time activities?	☐	☐	☐	☐
8. Were you short of breath?	☐	☐	☐	☐
9. Have you had pain?	☐	☐	☐	☐
10. Did you need to rest?	☐	☐	☐	☐
11. Have you had trouble sleeping?	☐	☐	☐	☐
12. Have you felt weak?	☐	☐	☐	☐
13. Have you lacked appetite?	☐	☐	☐	☐
14. Have you felt nauseated?	☐	☐	☐	☐

CRF nr: ☐☐☐☐

Please go to the next page

During the past week:	**Not at all**	**A little**	**Quite a bit**	**Very much**
15. Have you vomited?	☐	☐	☐	☐
16. Have you been constipated?	☐	☐	☐	☐
17. Have you had diarrhea?	☐	☐	☐	☐
18. Were you tired?	☐	☐	☐	☐
19. Did pain interfere with your daily activities?	☐	☐	☐	☐
20. Have you had difficulty in concentrating on things, like reading a newspaper or watching TV?	☐	☐	☐	☐
21. Did you feel tense?	☐	☐	☐	☐
22. Did you worry?	☐	☐	☐	☐
23. Did you feel irritable?	☐	☐	☐	☐
24. Did you feel depressed?	☐	☐	☐	☐
25. Have you had difficulty remembering things?	☐	☐	☐	☐
26. Has your physical condition or medical treatment interferred with your family life?	☐	☐	☐	☐
27. Has your physical condition or medical treatment interferred with your social activities?	☐	☐	☐	☐
28. Has your physical condition or medical treatment caused you financial difficulties?	☐	☐	☐	☐

For the following question please tick the number between 1 and 7 that best applies to you.

29. How would you rate your overall health during the past week?

☐ **1** ☐ **2** ☐ **3** ☐ **4** ☐ **5** ☐ **6** ☐ **7**

Very poor Excellent

30. How would you rate your overall quality of life during the past week?

☐ **1** ☐ **2** ☐ **3** ☐ **4** ☐ **5** ☐ **6** ☐ **7**

Very poor Excellent

CRF nr: | | | | |

NTNU DMF, IKM

Minimental status MMS

EAPC ONLUS

Projectno.:
OPI 03-006

1. ORIENTATION	Score	Maximum score
What year is it?	☐	1
What month is it?	☐	1
What season is it?	☐	1
What is today's date?	☐	1
What day of the week is it?	☐	1
What country are we in?	☐	1
What province/state/county are we in?	☐	1
What city/town are we in?	☐	1
What is the name of this hospital? (What is your homeadress?)	☐	1
What floor of the building are we on? (What is your postal code?)	☐	1
2. REGISTRATION Name 3 objects:1 second to say each. BALL - CAR - MAN:Then ask the patient all 3 after you have said them. Give 1 point for each correct answer. Then repeat them until he learns all 3. Count all trials ☐	☐	3
3. ATTENTION AND CALCULATION Spell "world" backwards 1 point for each correct. Alternatively: Ask patient to count backwards from 100 by sevens. Stop after 5 answers.	☐	5
4. RECALL Ask for the the 3 objects repeated above? Give 1 point for each correct.	☐	3
5. LANGUAGE		
Name a pencil	☐	1
Name a watch	☐	1
Repeat the following "No ifs, ands or buts."	☐	1
Follow a 3-stage command: Take a paper in your right hand, fold it in half, and put it on the floor."	☐	3
Read and obey the following: CLOSE YOUR EYES	☐	1
Write a sentence	☐	1
Copy design	☐	1
TOTAL SCORE	☐☐	30

CRF nr: ☐☐☐☐

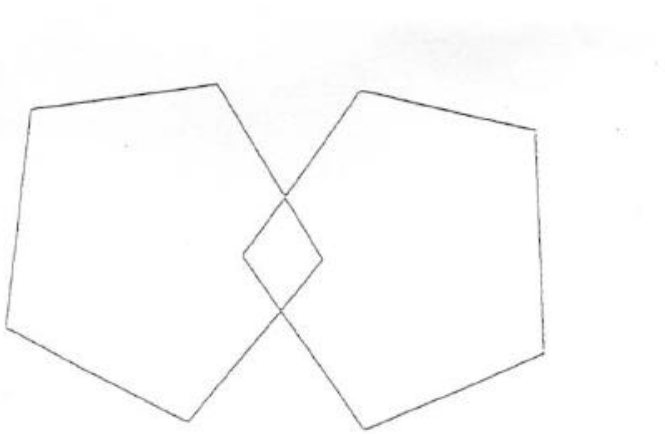
CLOSE YOUR EYES

CRF nr: | | | | |

Write a sentence

CRF nr: | | | | |

Copy design



CRF nr: | | | | |

Karnofsky Performance Scale

Criteria for functional status for patient with malign disease

100%	Normal, no complaints, no evidence of disease.
90%	Able to carry on normal activity: minor symptoms of disease.
80%	Normal activity with effort: some symptoms of disease.
70%	Cares for self: unable to carry on normal activity or active work.
60%	Requires occasional assistance but is able to care for needs.
50%	Requires considerable assistance and frequent medical care.
40%	Disabled: requires special care and assistance.
30%	Severely disabled: hospitalisation necessary: active treatment necessary.
20%	Very sick, hospitalisation necessary: active treatment necessary.
10%	Moribund, fatal processes progressing rapidly.

Dissertations at the Faculty of Medicine, NTNU

1977

1. Knut Joachim Berg: EFFECT OF ACETYLSALICYLIC ACID ON RENAL FUNCTION
2. Karl Erik Viken and Arne Ødegaard: STUDIES ON HUMAN MONOCYTES CULTURED *IN VITRO*

1978

3. Karel Bjørn Cyvin: CONGENITAL DISLOCATION OF THE HIP JOINT.
4. Alf O. Brubakk: METHODS FOR STUDYING FLOW DYNAMICS IN THE LEFT VENTRICLE AND THE AORTA IN MAN.

1979

5. Geirmund Unsgaard: CYTOSTATIC AND IMMUNOREGULATORY ABILITIES OF HUMAN BLOOD MONOCYTES CULTURED IN VITRO

1980

6. Størker Jørstad: URAEMIC TOXINS
7. Arne Olav Jenssen: SOME RHEOLOGICAL, CHEMICAL AND STRUCTURAL PROPERTIES OF MUCOID SPUTUM FROM PATIENTS WITH CHRONIC OBSTRUCTIVE BRONCHITIS

1981

8. Jens Hammerstrøm: CYTOSTATIC AND CYTOLYTIC ACTIVITY OF HUMAN MONOCYTES AND EFFUSION MACROPHAGES AGAINST TUMOR CELLS *IN VITRO*

1983

9. Tore Syversen: EFFECTS OF METHYLMERCURY ON RAT BRAIN PROTEIN.
10. Torbjørn Iversen: SQUAMOUS CELL CARCINOMA OF THE VULVA.

1984

11. Tor-Erik Widerøe: ASPECTS OF CONTINUOUS AMBULATORY PERITONEAL DIALYSIS.
12. Anton Hole: ALTERATIONS OF MONOCYTE AND LYMPHOCYTE FUNCTIONS IN REALTION TO SURGERY UNDER EPIDURAL OR GENERAL ANAESTHESIA.
13. Terje Terjesen: FRACTURE HEALING AND STRESS-PROTECTION AFTER METAL PLATE FIXATION AND EXTERNAL FIXATION.
14. Carsten Saunte: CLUSTER HEADACHE SYNDROME.
15. Inggard Lereim: TRAFFIC ACCIDENTS AND THEIR CONSEQUENCES.
16. Bjørn Magne Eggen: STUDIES IN CYTOTOXICITY IN HUMAN ADHERENT MONONUCLEAR BLOOD CELLS.
17. Trond Haug: FACTORS REGULATING BEHAVIORAL EFFECTS OG DRUGS.

1985

18. Sven Erik Gisvold: RESUSCITATION AFTER COMPLETE GLOBAL BRAIN ISCHEMIA.
19. Terje Espevik: THE CYTOSKELETON OF HUMAN MONOCYTES.
20. Lars Bevanger: STUDIES OF THE Ibc (c) PROTEIN ANTIGENS OF GROUP B STREPTOCOCCI.
21. Ole-Jan Iversen: RETROVIRUS-LIKE PARTICLES IN THE PATHOGENESIS OF PSORIASIS.
22. Lasse Eriksen: EVALUATION AND TREATMENT OF ALCOHOL DEPENDENT BEHAVIOUR.
23. Per I. Lundmo: ANDROGEN METABOLISM IN THE PROSTATE.

1986

24. Dagfinn Berntzen: ANALYSIS AND MANAGEMENT OF EXPERIMENTAL AND CLINICAL PAIN.
25. Odd Arnold Kildahl-Andersen: PRODUCTION AND CHARACTERIZATION OF MONOCYTE-DERIVED CYTOTOXIN AND ITS ROLE IN MONOCYTE-MEDIATED CYTOTOXICITY.
26. Ola Dale: VOLATILE ANAESTHETICS.

1987

27. Per Martin Kleveland: STUDIES ON GASTRIN.
28. Audun N. Øksendal: THE CALCIUM PARADOX AND THE HEART.
29. Vilhjalmur R. Finsen: HIP FRACTURES

1988

30. Rigmor Austgulen: TUMOR NECROSIS FACTOR: A MONOCYTE-DERIVED REGULATOR OF CELLULAR GROWTH.
31. Tom-Harald Edna: HEAD INJURIES ADMITTED TO HOSPITAL.
32. Joseph D. Borsi: NEW ASPECTS OF THE CLINICAL PHARMACOKINETICS OF METHOTREXATE.
33. Olav F. M. Sellevold: GLUCOCORTICOIDS IN MYOCARDIAL PROTECTION.
34. Terje Skjærpe: NONINVASIVE QUANTITATION OF GLOBAL PARAMETERS ON LEFT VENTRICULAR FUNCTION: THE SYSTOLIC PULMONARY ARTERY PRESSURE AND CARDIAC OUTPUT.
35. Eyvind Rødahl: STUDIES OF IMMUNE COMPLEXES AND RETROVIRUS-LIKE ANTIGENS IN PATIENTS WITH ANKYLOSING SPONDYLITIS.
36. Ketil Thorstensen: STUDIES ON THE MECHANISMS OF CELLULAR UPTAKE OF IRON FROM TRANSFERRIN.
37. Anna Midelfart: STUDIES OF THE MECHANISMS OF ION AND FLUID TRANSPORT IN THE BOVINE CORNEA.
38. Eirik Helseth: GROWTH AND PLASMINOGEN ACTIVATOR ACTIVITY OF HUMAN GLIOMAS AND BRAIN METASTASES - WITH SPECIAL REFERENCE TO TRANSFORMING GROWTH FACTOR BETA AND THE EPIDERMAL GROWTH FACTOR RECEPTOR.
39. Petter C. Borchgrevink: MAGNESIUM AND THE ISCHEMIC HEART.
40. Kjell-Arne Rein: THE EFFECT OF EXTRACORPOREAL CIRCULATION ON SUBCUTANEOUS TRANSCAPILLARY FLUID BALANCE.
41. Arne Kristian Sandvik: RAT GASTRIC HISTAMINE.
42. Carl Bredo Dahl: ANIMAL MODELS IN PSYCHIATRY.

1989

43. Torbjørn A. Fredriksen: CERVICOGENIC HEADACHE.
44. Rolf A. Walstad: CEFTAZIDIME.
45. Rolf Salvesen: THE PUPIL IN CLUSTER HEADACHE.
46. Nils Petter Jørgensen: DRUG EXPOSURE IN EARLY PREGNANCY.
47. Johan C. Ræder: PREMEDICATION AND GENERAL ANAESTHESIA IN OUTPATIENT GYNECOLOGICAL SURGERY.
48. M. R. Shalaby: IMMUNOREGULATORY PROPERTIES OF TNF-α AND THE RELATED CYTOKINES.
49. Anders Waage: THE COMPLEX PATTERN OF CYTOKINES IN SEPTIC SHOCK.
50. Bjarne Christian Eriksen: ELECTROSTIMULATION OF THE PELVIC FLOOR IN FEMALE URINARY INCONTINENCE.
51. Tore B. Halvorsen: PROGNOSTIC FACTORS IN COLORECTAL CANCER.

1990

52. Asbjørn Nordby: CELLULAR TOXICITY OF ROENTGEN CONTRAST MEDIA.
53. Kåre E. Tvedt: X-RAY MICROANALYSIS OF BIOLOGICAL MATERIAL.
54. Tore C. Stiles: COGNITIVE VULNERABILITY FACTORS IN THE DEVELOPMENT AND MAINTENANCE OF DEPRESSION.
55. Eva Hofsli: TUMOR NECROSIS FACTOR AND MULTIDRUG RESISTANCE.
56. Helge S. Haarstad: TROPHIC EFFECTS OF CHOLECYSTOKININ AND SECRETIN ON THE RAT PANCREAS.
57. Lars Engebretsen: TREATMENT OF ACUTE ANTERIOR CRUCIATE LIGAMENT INJURIES.
58. Tarjei Rygnestad: DELIBERATE SELF-POISONING IN TRONDHEIM.
59. Arne Z. Henriksen: STUDIES ON CONSERVED ANTIGENIC DOMAINS ON MAJOR OUTER MEMBRANE PROTEINS FROM ENTEROBACTERIA.
60. Steinar Westin: UNEMPLOYMENT AND HEALTH: Medical and social consequences of a factory closure in a ten-year controlled follow-up study.
61. Ylva Sahlin: INJURY REGISTRATION, a tool for accident preventive work.
62. Helge Bjørnstad Pettersen: BIOSYNTHESIS OF COMPLEMENT BY HUMAN ALVEOLAR MACROPHAGES WITH SPECIAL REFERENCE TO SARCOIDOSIS.
63. Berit Schei: TRAPPED IN PAINFUL LOVE.
64. Lars J. Vatten: PROSPECTIVE STUDIES OF THE RISK OF BREAST CANCER IN A COHORT OF NORWEGIAN WOMAN.

1991

65. Kåre Bergh: APPLICATIONS OF ANTI-C5a SPECIFIC MONOCLONAL ANTIBODIES FOR THE ASSESSMENT OF COMPLEMENT ACTIVATION.
66. Svein Svenningsen: THE CLINICAL SIGNIFICANCE OF INCREASED FEMORAL ANTEVERSION.
67. Olbjørn Klepp: NONSEMINOMATOUS GERM CELL TESTIS CANCER: THERAPEUTIC OUTCOME AND PROGNOSTIC FACTORS.
68. Trond Sand: THE EFFECTS OF CLICK POLARITY ON BRAINSTEM AUDITORY EVOKED POTENTIALS AMPLITUDE, DISPERSION, AND LATENCY VARIABLES.
69. Kjetil B. Åsbakk: STUDIES OF A PROTEIN FROM PSORIATIC SCALE, PSO P27, WITH RESPECT TO ITS POTENTIAL ROLE IN IMMUNE REACTIONS IN PSORIASIS.
70. Arnulf Hestnes: STUDIES ON DOWN´S SYNDROME.
71. Randi Nygaard: LONG-TERM SURVIVAL IN CHILDHOOD LEUKEMIA.
72. Bjørn Hagen: THIO-TEPA.
73. Svein Anda: EVALUATION OF THE HIP JOINT BY COMPUTED TOMOGRAMPHY AND ULTRASONOGRAPHY.

1992

74. Martin Svartberg: AN INVESTIGATION OF PROCESS AND OUTCOME OF SHORT-TERM PSYCHODYNAMIC PSYCHOTHERAPY.
75. Stig Arild Slørdahl: AORTIC REGURGITATION.
76. Harold C Sexton: STUDIES RELATING TO THE TREATMENT OF SYMPTOMATIC NON-PSYCHOTIC PATIENTS.
77. Maurice B. Vincent: VASOACTIVE PEPTIDES IN THE OCULAR/FOREHEAD AREA.
78. Terje Johannessen: CONTROLLED TRIALS IN SINGLE SUBJECTS.
79. Turid Nilsen: PYROPHOSPHATE IN HEPATOCYTE IRON METABOLISM.
80. Olav Haraldseth: NMR SPECTROSCOPY OF CEREBRAL ISCHEMIA AND REPERFUSION IN RAT.
81. Eiliv Brenna: REGULATION OF FUNCTION AND GROWTH OF THE OXYNTIC MUCOSA.

1993

82. Gunnar Bovim: CERVICOGENIC HEADACHE.
83. Jarl Arne Kahn: ASSISTED PROCREATION.
84. Bjørn Naume: IMMUNOREGULATORY EFFECTS OF CYTOKINES ON NK CELLS.
85. Rune Wiseth: AORTIC VALVE REPLACEMENT.
86. Jie Ming Shen: BLOOD FLOW VELOCITY AND RESPIRATORY STUDIES.
87. Piotr Kruszewski: SUNCT SYNDROME WITH SPECIAL REFERENCE TO THE AUTONOMIC NERVOUS SYSTEM.
88. Mette Haase Moen: ENDOMETRIOSIS.
89. Anne Vik: VASCULAR GAS EMBOLISM DURING AIR INFUSION AND AFTER DECOMPRESSION IN PIGS.
90. Lars Jacob Stovner: THE CHIARI TYPE I MALFORMATION.
91. Kjell Å. Salvesen: ROUTINE ULTRASONOGRAPHY IN UTERO AND DEVELOPMENT IN CHILDHOOD.

1994

92. Nina-Beate Liabakk: DEVELOPMENT OF IMMUNOASSAYS FOR TNF AND ITS SOLUBLE RECEPTORS.
93. Sverre Helge Torp: *erb*B ONCOGENES IN HUMAN GLIOMAS AND MENINGIOMAS.
94. Olav M. Linaker: MENTAL RETARDATION AND PSYCHIATRY. Past and present.
95. Per Oscar Feet: INCREASED ANTIDEPRESSANT AND ANTIPANIC EFFECT IN COMBINED TREATMENT WITH DIXYRAZINE AND TRICYCLIC ANTIDEPRESSANTS.
96. Stein Olav Samstad: CROSS SECTIONAL FLOW VELOCITY PROFILES FROM TWO-DIMENSIONAL DOPPLER ULTRASOUND: Studies on early mitral blood flow.
97. Bjørn Backe: STUDIES IN ANTENATAL CARE.
98. Gerd Inger Ringdal: QUALITY OF LIFE IN CANCER PATIENTS.
99. Torvid Kiserud: THE DUCTUS VENOSUS IN THE HUMAN FETUS.
100. Hans E. Fjøsne: HORMONAL REGULATION OF PROSTATIC METABOLISM.
101. Eylert Brodtkorb: CLINICAL ASPECTS OF EPILEPSY IN THE MENTALLY RETARDED.
102. Roar Juul: PEPTIDERGIC MECHANISMS IN HUMAN SUBARACHNOID HEMORRHAGE.
103. Unni Syversen: CHROMOGRANIN A. Phsysiological and Clinical Role.

1995

104. Odd Gunnar Brakstad: THERMOSTABLE NUCLEASE AND THE *nuc* GENE IN THE DIAGNOSIS OF *Staphylococcus aureus* INFECTIONS.
105. Terje Engan: NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY OF PLASMA IN MALIGNANT DISEASE.
106. Kirsten Rasmussen: VIOLENCE IN THE MENTALLY DISORDERED.
107. Finn Egil Skjeldestad: INDUCED ABORTION: Timetrends and Determinants.
108. Roar Stenseth: THORACIC EPIDURAL ANALGESIA IN AORTOCORONARY BYPASS SURGERY.
109. Arild Faxvaag: STUDIES OF IMMUNE CELL FUNCTION *in mice infected with* MURINE RETROVIRUS.

1996

110. Svend Aakhus: NONINVASIVE COMPUTERIZED ASSESSMENT OF LEFT VENTRICULAR FUNCTION AND SYSTEMIC ARTERIAL PROPERTIES. Methodology and some clinical applications.
111. Klaus-Dieter Bolz: INTRAVASCULAR ULTRASONOGRAPHY.
112. Petter Aadahl: CARDIOVASCULAR EFFECTS OF THORACIC AORTIC CROSS-CLAMPING.
113. Sigurd Steinshamn: CYTOKINE MEDIATORS DURING GRANULOCYTOPENIC INFECTIONS.
114. Hans Stifoss-Hanssen: SEEKING MEANING OR HAPPINESS?
115. Anne Kvikstad: LIFE CHANGE EVENTS AND MARITAL STATUS IN RELATION TO RISK AND PROGNOSIS OF CANCER.
116. Torbjørn Grøntvedt: TREATMENT OF ACUTE AND CHRONIC ANTERIOR CRUCIATE LIGAMENT INJURIES. A clinical and biomechanical study.
117. Sigrid Hørven Wigers: CLINICAL STUDIES OF FIBROMYALGIA WITH FOCUS ON ETIOLOGY, TREATMENT AND OUTCOME.
118. Jan Schjøtt: MYOCARDIAL PROTECTION: Functional and Metabolic Characteristics of Two Endogenous Protective Principles.
119. Marit Martinussen: STUDIES OF INTESTINAL BLOOD FLOW AND ITS RELATION TO TRANSITIONAL CIRCULATORY ADAPATION IN NEWBORN INFANTS.
120. Tomm B. Müller: MAGNETIC RESONANCE IMAGING IN FOCAL CEREBRAL ISCHEMIA.
121. Rune Haaverstad: OEDEMA FORMATION OF THE LOWER EXTREMITIES.
122. Magne Børset: THE ROLE OF CYTOKINES IN MULTIPLE MYELOMA, WITH SPECIAL REFERENCE TO HEPATOCYTE GROWTH FACTOR.
123. Geir Smedslund: A THEORETICAL AND EMPIRICAL INVESTIGATION OF SMOKING, STRESS AND DISEASE: RESULTS FROM A POPULATION SURVEY.

1997

124. Torstein Vik: GROWTH, MORBIDITY, AND PSYCHOMOTOR DEVELOPMENT IN INFANTS WHO WERE GROWTH RETARDED *IN UTERO*.
125. Siri Forsmo: ASPECTS AND CONSEQUENCES OF OPPORTUNISTIC SCREENING FOR CERVICAL CANCER. Results based on data from three Norwegian counties.
126. Jon S. Skranes: CEREBRAL MRI AND NEURODEVELOPMENTAL OUTCOME IN VERY LOW BIRTH WEIGHT (VLBW) CHILDREN. A follow-up study of a geographically based year cohort of VLBW children at ages one and six years.
127. Knut Bjørnstad: COMPUTERIZED ECHOCARDIOGRAPHY FOR EVALUTION OF CORONARY ARTERY DISEASE.
128. Grethe Elisabeth Borchgrevink: DIAGNOSIS AND TREATMENT OF WHIPLASH/NECK SPRAIN INJURIES CAUSED BY CAR ACCIDENTS.
129. Tor Elsås: NEUROPEPTIDES AND NITRIC OXIDE SYNTHASE IN OCULAR AUTONOMIC AND SENSORY NERVES.
130. Rolf W. Gråwe: EPIDEMIOLOGICAL AND NEUROPSYCHOLOGICAL PERSPECTIVES ON SCHIZOPHRENIA.
131. Tonje Strømholm: CEREBRAL HAEMODYNAMICS DURING THORACIC AORTIC CROSSCLAMPING. An experimental study in pigs.

1998

132. Martinus Bråten: STUDIES ON SOME PROBLEMS REALTED TO INTRAMEDULLARY NAILING OF FEMORAL FRACTURES.
133. Ståle Nordgård: PROLIFERATIVE ACTIVITY AND DNA CONTENT AS PROGNOSTIC INDICATORS IN ADENOID CYSTIC CARCINOMA OF THE HEAD AND NECK.

134. Egil Lien: SOLUBLE RECEPTORS FOR **TNF** AND **LPS**: RELEASE PATTERN AND POSSIBLE SIGNIFICANCE IN DISEASE.
135. Marit Bjørgaas: HYPOGLYCAEMIA IN CHILDREN WITH DIABETES MELLITUS
136. Frank Skorpen: GENETIC AND FUNCTIONAL ANALYSES OF DNA REPAIR IN HUMAN CELLS.
137. Juan A. Pareja: SUNCT SYNDROME. ON THE CLINICAL PICTURE. ITS DISTINCTION FROM OTHER, SIMILAR HEADACHES.
138. Anders Angelsen: NEUROENDOCRINE CELLS IN HUMAN PROSTATIC CARCINOMAS AND THE PROSTATIC COMPLEX OF RAT, GUINEA PIG, CAT AND DOG.
139. Fabio Antonaci: CHRONIC PAROXYSMAL HEMICRANIA AND HEMICRANIA CONTINUA: TWO DIFFERENT ENTITIES?
140. Sven M. Carlsen: ENDOCRINE AND METABOLIC EFFECTS OF METFORMIN WITH SPECIAL EMPHASIS ON CARDIOVASCULAR RISK FACTORES.

1999

141. Terje A. Murberg: DEPRESSIVE SYMPTOMS AND COPING AMONG PATIENTS WITH CONGESTIVE HEART FAILURE.
142. Harm-Gerd Karl Blaas: THE EMBRYONIC EXAMINATION. Ultrasound studies on the development of the human embryo.
143. Noèmi Becser Andersen:THE CEPHALIC SENSORY NERVES IN UNILATERAL HEADACHES. Anatomical background and neurophysiological evaluation.
144. Eli-Janne Fiskerstrand: LASER TREATMENT OF PORT WINE STAINS. A study of the efficacy and limitations of the pulsed dye laser. Clinical and morfological analyses aimed at improving the therapeutic outcome.
145. Bård Kulseng: A STUDY OF ALGINATE CAPSULE PROPERTIES AND CYTOKINES IN RELATION TO INSULIN DEPENDENT DIABETES MELLITUS.
146. Terje Haug: STRUCTURE AND REGULATION OF THE HUMAN UNG GENE ENCODING URACIL-DNA GLYCOSYLASE.
147. Heidi Brurok: MANGANESE AND THE HEART. A Magic Metal with Diagnostic and Therapeutic Possibilites.
148. Agnes Kathrine Lie: DIAGNOSIS AND PREVALENCE OF HUMAN PAPILLOMAVIRUS INFECTION IN CERVICAL INTRAEPITELIAL NEOPLASIA. Relationship to Cell Cycle Regulatory Proteins and HLA DQBI Genes.
149. Ronald Mårvik: PHARMACOLOGICAL, PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL STUDIES ON ISOLATED STOMACS.
150. Ketil Jarl Holen: THE ROLE OF ULTRASONOGRAPHY IN THE DIAGNOSIS AND TREATMENT OF HIP DYSPLASIA IN NEWBORNS.
151. Irene Hetlevik: THE ROLE OF CLINICAL GUIDELINES IN CARDIOVASCULAR RISK INTERVENTION IN GENERAL PRACTICE.
152. Katarina Tunòn: ULTRASOUND AND PREDICTION OF GESTATIONAL AGE.
153. Johannes Soma: INTERACTION BETWEEN THE LEFT VENTRICLE AND THE SYSTEMIC ARTERIES.
154. Arild Aamodt: DEVELOPMENT AND PRE-CLINICAL EVALUATION OF A CUSTOM-MADE FEMORAL STEM.
155. Agnar Tegnander: DIAGNOSIS AND FOLLOW-UP OF CHILDREN WITH SUSPECTED OR KNOWN HIP DYSPLASIA.
156. Bent Indredavik: STROKE UNIT TREATMENT: SHORT AND LONG-TERM EFFECTS
157. Jolanta Vanagaite Vingen: PHOTOPHOBIA AND PHONOPHOBIA IN PRIMARY HEADACHES

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158. Ola Dalsegg Sæther: PATHOPHYSIOLOGY DURING PROXIMAL AORTIC CROSS-CLAMPING CLINICAL AND EXPERIMENTAL STUDIES
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160. Christina Vogt Isaksen: PRENATAL ULTRASOUND AND POSTMORTEM FINDINGS – A TEN YEAR CORRELATIVE STUDY OF FETUSES AND INFANTS WITH DEVELOPMENTAL ANOMALIES.
161. Holger Seidel: HIGH-DOSE METHOTREXATE THERAPY IN CHILDREN WITH ACUTE LYMPHOCYTIC LEUKEMIA: DOSE, CONCENTRATION, AND EFFECT CONSIDERATIONS.
162. Stein Hallan: IMPLEMENTATION OF MODERN MEDICAL DECISION ANALYSIS INTO CLINICAL DIAGNOSIS AND TREATMENT.

163. Malcolm Sue-Chu: INVASIVE AND NON-INVASIVE STUDIES IN CROSS-COUNTRY SKIERS WITH ASTHMA-LIKE SYMPTOMS.
164. Ole-Lars Brekke: EFFECTS OF ANTIOXIDANTS AND FATTY ACIDS ON TUMOR NECROSIS FACTOR-INDUCED CYTOTOXICITY.
165. Jan Lundbom: AORTOCORONARY BYPASS SURGERY: CLINICAL ASPECTS, COST CONSIDERATIONS AND WORKING ABILITY.
166. John-Anker Zwart: LUMBAR NERVE ROOT COMPRESSION, BIOCHEMICAL AND NEUROPHYSIOLOGICAL ASPECTS.
167. Geir Falck: HYPEROSMOLALITY AND THE HEART.
168. Eirik Skogvoll: CARDIAC ARREST Incidence, Intervention and Outcome.
169. Dalius Bansevicius: SHOULDER-NECK REGION IN CERTAIN HEADACHES AND CHRONIC PAIN SYNDROMES.
170. Bettina Kinge: REFRACTIVE ERRORS AND BIOMETRIC CHANGES AMONG UNIVERSITY STUDENTS IN NORWAY.
171. Gunnar Qvigstad: CONSEQUENCES OF HYPERGASTRINEMIA IN MAN
172. Hanne Ellekjær: EPIDEMIOLOGICAL STUDIES OF STROKE IN A NORWEGIAN POPULATION. INCIDENCE, RISK FACTORS AND PROGNOSIS
173. Hilde Grimstad: VIOLENCE AGAINST WOMEN AND PREGNANCY OUTCOME.
174. Astrid Hjelde: SURFACE TENSION AND COMPLEMENT ACTIVATION: Factors influencing bubble formation and bubble effects after decompression.
175. Kjell A. Kvistad: MR IN BREAST CANCER – A CLINICAL STUDY.
176. Ivar Rossvoll: ELECTIVE ORTHOPAEDIC SURGERY IN A DEFINED POPULATION. Studies on demand, waiting time for treatment and incapacity for work.
177. Carina Seidel: PROGNOSTIC VALUE AND BIOLOGICAL EFFECTS OF HEPATOCYTE GROWTH FACTOR AND SYNDECAN-1 IN MULTIPLE MYELOMA.

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178. Alexander Wahba: THE INFLUENCE OF CARDIOPULMONARY BYPASS ON PLATELET FUNCTION AND BLOOD COAGULATION – DETERMINANTS AND CLINICAL CONSEQUENSES
179. Marcus Schmitt-Egenolf: THE RELEVANCE OF THE MAJOR hISTOCOMPATIBILITY COMPLEX FOR THE GENETICS OF PSORIASIS
180. Odrun Arna Gederaas: BIOLOGICAL MECHANISMS INVOLVED IN 5-AMINOLEVULINIC ACID BASED PHOTODYNAMIC THERAPY
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183. Gunnar Morken: SEASONAL VARIATION OF HUMAN MOOD AND BEHAVIOUR
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186. Knut Ivar Aasarød: RENAL INVOLVEMENT IN INFLAMMATORY RHEUMATIC DISEASE. A Study of Renal Disease in Wegener's Granulomatosis and in Primary Sjögren's Syndrome
187. Trude Helen Flo: RESEPTORS INVOLVED IN CELL ACTIVATION BY DEFINED URONIC ACID POLYMERS AND BACTERIAL COMPONENTS
188. Bodil Kavli: HUMAN URACIL-DNA GLYCOSYLASES FROM THE UNG GENE: STRUCTRUAL BASIS FOR SUBSTRATE SPECIFICITY AND REPAIR
189. Liv Thommesen: MOLECULAR MECHANISMS INVOLVED IN TNF- AND GASTRIN-MEDIATED GENE REGULATION
190. Turid Lingaas Holmen: SMOKING AND HEALTH IN ADOLESCENCE; THE NORD-TRØNDELAG HEALTH STUDY, 1995-97
191. Øyvind Hjertner: MULTIPLE MYELOMA: INTERACTIONS BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MICROENVIRONMENT
192. Asbjørn Støylen: STRAIN RATE IMAGING OF THE LEFT VENTRICLE BY ULTRASOUND. FEASIBILITY, CLINICAL VALIDATION AND PHYSIOLOGICAL ASPECTS

193. Kristian Midthjell: DIABETES IN ADULTS IN NORD-TRØNDELAG. PUBLIC HEALTH ASPECTS OF DIABETES MELLITUS IN A LARGE, NON-SELECTED NORWEGIAN POPULATION.
194. Guanglin Cui: FUNCTIONAL ASPECTS OF THE ECL CELL IN RODENTS
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197. Tore Amundsen: PERFUSION MR IMAGING IN THE DIAGNOSIS OF PULMONARY EMBOLISM
198. Nanna Kurtze: THE SIGNIFICANCE OF ANXIETY AND DEPRESSION IN FATIQUE AND PATTERNS OF PAIN AMONG INDIVIDUALS DIAGNOSED WITH FIBROMYALGIA: RELATIONS WITH QUALITY OF LIFE, FUNCTIONAL DISABILITY, LIFESTYLE, EMPLOYMENT STATUS, CO-MORBIDITY AND GENDER
199. Tom Ivar Lund Nilsen: PROSPECTIVE STUDIES OF CANCER RISK IN NORD-TRØNDELAG: THE HUNT STUDY. Associations with anthropometric, socioeconomic, and lifestyle risk factors
200. Asta Kristine Håberg: A NEW APPROACH TO THE STUDY OF MIDDLE CEREBRAL ARTERY OCCLUSION IN THE RAT USING MAGNETIC RESONANCE TECHNIQUES

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201. Knut Jørgen Arntzen: PREGNANCY AND CYTOKINES
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203. Asta Bye: LOW FAT, LOW LACTOSE DIET USED AS PROPHYLACTIC TREATMENT OF ACUTE INTESTINAL REACTIONS DURING PELVIC RADIOTHERAPY. A PROSPECTIVE RANDOMISED STUDY.
204. Sylvester Moyo: STUDIES ON STREPTOCOCCUS AGALACTIAE (GROUP B STREPTOCOCCUS) SURFACE-ANCHORED MARKERS WITH EMPHASIS ON STRAINS AND HUMAN SERA FROM ZIMBABWE.
205. Knut Hagen: HEAD-HUNT: THE EPIDEMIOLOGY OF HEADACHE IN NORD-TRØNDELAG
206. Li Lixin: ON THE REGULATION AND ROLE OF UNCOUPLING PROTEIN-2 IN INSULIN PRODUCING ß-CELLS
207. Anne Hildur Henriksen: SYMPTOMS OF ALLERGY AND ASTHMA VERSUS MARKERS OF LOWER AIRWAY INFLAMMATION AMONG ADOLESCENTS
208. Egil Andreas Fors: NON-MALIGNANT PAIN IN RELATION TO PSYCHOLOGICAL AND ENVIRONTENTAL FACTORS. EXPERIENTAL AND CLINICAL STUDES OF PAIN WITH FOCUS ON FIBROMYALGIA
209. Pål Klepstad: MORPHINE FOR CANCER PAIN
210. Ingunn Bakke: MECHANISMS AND CONSEQUENCES OF PEROXISOME PROLIFERATOR-INDUCED HYPERFUNCTION OF THE RAT GASTRIN PRODUCING CELL
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212. Rønnaug Astri Ødegård: PREECLAMPSIA – MATERNAL RISK FACTORS AND FETAL GROWTH
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214. Turid Suzanne Berg-Nielsen: PARENTING PRACTICES AND MENTALLY DISORDERED ADOLESCENTS
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216. Jan Pål Loennechen: HEART FAILURE AFTER MYOCARDIAL INFARCTION. Regional Differences, Myocyte Function, Gene Expression, and Response to Cariporide, Losartan, and Exercise Training.
217. Elisabeth Qvigstad: EFFECTS OF FATTY ACIDS AND OVER-STIMULATION ON INSULIN SECRETION IN MAN

218. Arne Åsberg: EPIDEMIOLOGICAL STUDIES IN HEREDITARY HEMOCHROMATOSIS: PREVALENCE, MORBIDITY AND BENEFIT OF SCREENING.
219. Johan Fredrik Skomsvoll: REPRODUCTIVE OUTCOME IN WOMEN WITH RHEUMATIC DISEASE. A population registry based study of the effects of inflammatory rheumatic disease and connective tissue disease on reproductive outcome in Norwegian women in 1967-1995.
220. Siv Mørkved: URINARY INCONTINENCE DURING PREGNANCY AND AFTER DELIVERY: EFFECT OF PELVIC FLOOR MUSCLE TRAINING IN PREVENTION AND TREATMENT
221. Marit S. Jordhøy: THE IMPACT OF COMPREHENSIVE PALLIATIVE CARE
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223. Solveig Tingulstad: CENTRALIZATION OF PRIMARY SURGERY FOR OVARAIN CANCER. FEASIBILITY AND IMPACT ON SURVIVAL
224. Haytham Eloqayli: METABOLIC CHANGES IN THE BRAIN CAUSED BY EPILEPTIC SEIZURES
225. Torunn Bruland: STUDIES OF EARLY RETROVIRUS-HOST INTERACTIONS – VIRAL DETERMINANTS FOR PATHOGENESIS AND THE INFLUENCE OF SEX ON THE SUSCEPTIBILITY TO FRIEND MURINE LEUKAEMIA VIRUS INFECTION
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227. Vibeke Nossum: THE EFFECT OF VASCULAR BUBBLES ON ENDOTHELIAL FUNCTION
228. Sigurd Fasting: ROUTINE BASED RECORDING OF ADVERSE EVENTS DURING ANAESTHESIA – APPLICATION IN QUALITY IMPROVEMENT AND SAFETY
229. Solfrid Romundstad: EPIDEMIOLOGICAL STUDIES OF MICROALBUMINURIA. THE NORD-TRØNDELAG HEALTH STUDY 1995-97 (HUNT 2)
230. Geir Torheim: PROCESSING OF DYNAMIC DATA SETS IN MAGNETIC RESONANCE IMAGING
231. Catrine Ahlén: SKIN INFECTIONS IN OCCUPATIONAL SATURATION DIVERS IN THE NORTH SEA AND THE IMPACT OF THE ENVIRONMENT
232. Arnulf Langhammer: RESPIRATORY SYMPTOMS, LUNG FUNCTION AND BONE MINERAL DENSITY IN A COMPREHENSIVE POPULATION SURVEY. THE NORD-TRØNDELAG HEALTH STUDY 1995-97. THE BRONCHIAL OBSTRUCTION IN NORD-TRØNDELAG STUDY
233. Einar Kjelsås: EATING DISORDERS AND PHYSICAL ACTIVITY IN NON-CLINICAL SAMPLES
234. Arne Wibe: RECTAL CANCER TREATMENT IN NORWAY – STANDARDISATION OF SURGERY AND QUALITY ASSURANCE

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235. Eivind Witsø: BONE GRAFT AS AN ANTIBIOTIC CARRIER
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237. Hallvard Lærum: EVALUATION OF ELECTRONIC MEDICAL RECORDS – A CLINICAL TASK PERSPECTIVE
238. Gustav Mikkelsen: ACCESSIBILITY OF INFORMATION IN ELECTRONIC PATIENT RECORDS; AN EVALUATION OF THE ROLE OF DATA QUALITY
239. Steinar Krokstad: SOCIOECONOMIC INEQUALITIES IN HEALTH AND DISABILITY. SOCIAL EPIDEMIOLOGY IN THE NORD-TRØNDELAG HEALTH STUDY (HUNT), NORWAY
240. Arne Kristian Myhre: NORMAL VARIATION IN ANOGENITAL ANATOMY AND MICROBIOLOGY IN NON-ABUSED PRESCHOOL CHILDREN
241. Ingunn Dybedal: NEGATIVE REGULATORS OF HEMATOPOIETEC STEM AND PROGENITOR CELLS
242. Beate Sitter: TISSUE CHARACTERIZATION BY HIGH RESOLUTION MAGIC ANGLE SPINNING MR SPECTROSCOPY
243. Per Arne Aas: MACROMOLECULAR MAINTENANCE IN HUMAN CELLS – REPAIR OF URACIL IN DNA AND METHYLATIONS IN DNA AND RNA
244. Anna Bofin: FINE NEEDLE ASPIRATION CYTOLOGY IN THE PRIMARY INVESTIGATION OF BREAST TUMOURS AND IN THE DETERMINATION OF TREATMENT STRATEGIES

245. Jim Aage Nøttestad: DEINSTITUTIONALIZATION AND MENTAL HEALTH CHANGES AMONG PEOPLE WITH MENTAL RETARDATION
246. Reidar Fossmark: GASTRIC CANCER IN JAPANESE COTTON RATS
247. Wibeke Nordhøy: MANGANESE AND THE HEART, INTRACELLULAR MR RELAXATION AND WATER EXCHANGE ACROSS THE CARDIAC CELL MEMBRANE

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248. Sturla Molden: QUANTITATIVE ANALYSES OF SINGLE UNITS RECORDED FROM THE HIPPOCAMPUS AND ENTORHINAL CORTEX OF BEHAVING RATS
249. Wenche Brenne Drøyvold: EPIDEMIOLOGICAL STUDIES ON WEIGHT CHANGE AND HEALTH IN A LARGE POPULATION. THE NORD-TRØNDELAG HEALTH STUDY (HUNT)
250. Ragnhild Støen: ENDOTHELIUM-DEPENDENT VASODILATION IN THE FEMORAL ARTERY OF DEVELOPING PIGLETS
251. Aslak Steinsbekk: HOMEOPATHY IN THE PREVENTION OF UPPER RESPIRATORY TRACT INFECTIONS IN CHILDREN
252. Hill-Aina Steffenach: MEMORY IN HIPPOCAMPAL AND CORTICO-HIPPOCAMPAL CIRCUITS
253. Eystein Stordal: ASPECTS OF THE EPIDEMIOLOGY OF DEPRESSIONS BASED ON SELF-RATING IN A LARGE GENERAL HEALTH STUDY (THE HUNT-2 STUDY)
254. Viggo Pettersen: FROM MUSCLES TO SINGING: THE ACTIVITY OF ACCESSORY BREATHING MUSCLES AND THORAX MOVEMENT IN CLASSICAL SINGING
255. Marianne Fyhn: SPATIAL MAPS IN THE HIPPOCAMPUS AND ENTORHINAL CORTEX
256. Robert Valderhaug: OBSESSIVE-COMPULSIVE DISORDER AMONG CHILDREN AND ADOLESCENTS: CHARACTERISTICS AND PSYCHOLOGICAL MANAGEMENT OF PATIENTS IN OUTPATIENT PSYCHIATRIC CLINICS
257. Erik Skaaheim Haug: INFRARENAL ABDOMINAL AORTIC ANEURYSMS – COMORBIDITY AND RESULTS FOLLOWING OPEN SURGERY
258. Daniel Kondziella: GLIAL-NEURONAL INTERACTIONS IN EXPERIMENTAL BRAIN DISORDERS
259. Vegard Heimly Brun: ROUTES TO SPATIAL MEMORY IN HIPPOCAMPAL PLACE CELLS
260. Kenneth McMillan: PHYSIOLOGICAL ASSESSMENT AND TRAINING OF ENDURANCE AND STRENGTH IN PROFESSIONAL YOUTH SOCCER PLAYERS
261. Marit Sæbø Indredavik: MENTAL HEALTH AND CEREBRAL MAGNETIC RESONANCE IMAGING IN ADOLESCENTS WITH LOW BIRTH WEIGHT
262. Ole Johan Kemi: ON THE CELLULAR BASIS OF AEROBIC FITNESS, INTENSITY-DEPENDENCE AND TIME-COURSE OF CARDIOMYOCYTE AND ENDOTHELIAL ADAPTATIONS TO EXERCISE TRAINING
263. Eszter Vanky: POLYCYSTIC OVARY SYNDROME – METFORMIN TREATMENT IN PREGNANCY
264. Hild Fjærtoft: EXTENDED STROKE UNIT SERVICE AND EARLY SUPPORTED DISCHARGE. SHORT AND LONG-TERM EFFECTS
265. Grete Dyb: POSTTRAUMATIC STRESS REACTIONS IN CHILDREN AND ADOLESCENTS
266. Vidar Fykse: SOMATOSTATIN AND THE STOMACH
267. Kirsti Berg: OXIDATIVE STRESS AND THE ISCHEMIC HEART: A STUDY IN PATIENTS UNDERGOING CORONARY REVASCULARIZATION
268. Björn Inge Gustafsson: THE SEROTONIN PRODUCING ENTEROCHROMAFFIN CELL, AND EFFECTS OF HYPERSEROTONINEMIA ON HEART AND BONE

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269. Torstein Baade Rø: EFFECTS OF BONE MORPHOGENETIC PROTEINS, HEPATOCYTE GROWTH FACTOR AND INTERLEUKIN-21 IN MULTIPLE MYELOMA
270. May-Britt Tessem: METABOLIC EFFECTS OF ULTRAVIOLET RADIATION ON THE ANTERIOR PART OF THE EYE
271. Anne-Sofie Helvik: COPING AND EVERYDAY LIFE IN A POPULATION OF ADULTS WITH HEARING IMPAIRMENT
272. Therese Standal: MULTIPLE MYELOMA: THE INTERPLAY BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MARROW MICROENVIRONMENT

273. Ingvild Saltvedt: TREATMENT OF ACUTELY SICK, FRAIL ELDERLY PATIENTS IN A GERIATRIC EVALUATION AND MANAGEMENT UNIT – RESULTS FROM A PROSPECTIVE RANDOMISED TRIAL
274. Birger Henning Endreseth: STRATEGIES IN RECTAL CANCER TREATMENT – FOCUS ON EARLY RECTAL CANCER AND THE INFLUENCE OF AGE ON PROGNOSIS
275. Anne Mari Aukan Rokstad: ALGINATE CAPSULES AS BIOREACTORS FOR CELL THERAPY
276. Mansour Akbari: HUMAN BASE EXCISION REPAIR FOR PRESERVATION OF GENOMIC STABILITY
277. Stein Sundstrøm: IMPROVING TREATMENT IN PATIENTS WITH LUNG CANCER – RESULTS FROM TWO MULITCENTRE RANDOMISED STUDIES
278. Hilde Pleym: BLEEDING AFTER CORONARY ARTERY BYPASS SURGERY - STUDIES ON HEMOSTATIC MECHANISMS, PROPHYLACTIC DRUG TREATMENT AND EFFECTS OF AUTOTRANSFUSION
279. Line Merethe Oldervoll: PHYSICAL ACTIVITY AND EXERCISE INTERVENTIONS IN CANCER PATIENTS
280. Boye Welde: THE SIGNIFICANCE OF ENDURANCE TRAINING, RESISTANCE TRAINING AND MOTIVATIONAL STYLES IN ATHLETIC PERFORMANCE AMONG ELITE JUNIOR CROSS-COUNTRY SKIERS
281. Per Olav Vandvik: IRRITABLE BOWEL SYNDROME IN NORWAY, STUDIES OF PREVALENCE, DIAGNOSIS AND CHARACTERISTICS IN GENERAL PRACTICE AND IN THE POPULATION
282. Idar Kirkeby-Garstad: CLINICAL PHYSIOLOGY OF EARLY MOBILIZATION AFTER CARDIAC SURGERY
283. Linn Getz: SUSTAINABLE AND RESPONSIBLE PREVENTIVE MEDICINE. CONCEPTUALISING ETHICAL DILEMMAS ARISING FROM CLINICAL IMPLEMENTATION OF ADVANCING MEDICAL TECHNOLOGY
284. Eva Tegnander: DETECTION OF CONGENITAL HEART DEFECTS IN A NON-SELECTED POPULATION OF 42,381 FETUSES
285. Kristin Gabestad Nørsett: GENE EXPRESSION STUDIES IN GASTROINTESTINAL PATHOPHYSIOLOGY AND NEOPLASIA
286. Per Magnus Haram: GENETIC VS. AQUIRED FITNESS: METABOLIC, VASCULAR AND CARDIOMYOCYTE ADAPTATIONS
287. Agneta Johansson: GENERAL RISK FACTORS FOR GAMBLING PROBLEMS AND THE PREVALENCE OF PATHOLOGICAL GAMBLING IN NORWAY
288. Svein Artur Jensen: THE PREVALENCE OF SYMPTOMATIC ARTERIAL DISEASE OF THE LOWER LIMB
289. Charlotte Björk Ingul: QUANITIFICATION OF REGIONAL MYOCARDIAL FUNCTION BY STRAIN RATE AND STRAIN FOR EVALUATION OF CORONARY ARTERY DISEASE. AUTOMATED VERSUS MANUAL ANALYSIS DURING ACUTE MYOCARDIAL INFARCTION AND DOBUTAMINE STRESS ECHOCARDIOGRAPHY
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292. Ottar Bjerkeset: ANXIETY AND DEPRESSION IN THE GENERAL POPULATION: RISK FACTORS, INTERVENTION AND OUTCOME – THE NORD-TRØNDELAG HEALTH STUDY (HUNT)
293. Jon Olav Drogset: RESULTS AFTER SURGICAL TREATMENT OF ANTERIOR CRUCIATE LIGAMENT INJURIES – A CLINICAL STUDY
294. Lars Fosse: MECHANICAL BEHAVIOUR OF COMPACTED MORSELLISED BONE – AN EXPERIMENTAL IN VITRO STUDY
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296. Paul Jarle Mork: MUSCLE ACTIVITY IN WORK AND LEISURE AND ITS ASSOCIATION TO MUSCULOSKELETAL PAIN
297. Björn Stenström: LESSONS FROM RODENTS: I: MECHANISMS OF OBESITY SURGERY – ROLE OF STOMACH. II: CARCINOGENIC EFFECTS OF *HELICOBACTER PYLORI* AND SNUS IN THE STOMACH

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300. May Britt Drugli: YOUNG CHILDREN TREATED BECAUSE OF ODD/CD: CONDUCT PROBLEMS AND SOCIAL COMPETENCIES IN DAY-CARE AND SCHOOL SETTINGS
301. Arne Skjold: MAGNETIC RESONANCE KINETICS OF MANGANESE DIPYRIDOXYL DIPHOSPHATE (MnDPDP) IN HUMAN MYOCARDIUM. STUDIES IN HEALTHY VOLUNTEERS AND IN PATIENTS WITH RECENT MYOCARDIAL INFARCTION
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305. Elisabeth Olstad: GLUTAMATE AND GABA: MAJOR PLAYERS IN NEURONAL METABOLISM
306. Lilian Leistad: THE ROLE OF CYTOKINES AND PHOSPHOLIPASE A_2S IN ARTICULAR CARTILAGE CHONDROCYTES IN RHEUMATOID ARTHRITIS AND OSTEOARTHRITIS
307. Arne Vaaler: EFFECTS OF PSYCHIATRIC INTENSIVE CARE UNIT IN AN ACUTE PSYCIATHRIC WARD
308. Mathias Toft: GENETIC STUDIES OF LRRK2 AND PINK1 IN PARKINSON'S DISEASE
309. Ingrid Løvold Mostad: IMPACT OF DIETARY FAT QUANTITY AND QUALITY IN TYPE 2 DIABETES WITH EMPHASIS ON MARINE N-3 FATTY ACIDS
310. Torill Eidhammer Sjøbakk: MR DETERMINED BRAIN METABOLIC PATTERN IN PATIENTS WITH BRAIN METASTASES AND ADOLESCENTS WITH LOW BIRTH WEIGHT
311. Vidar Beisvåg: PHYSIOLOGICAL GENOMICS OF HEART FAILURE: FROM TECHNOLOGY TO PHYSIOLOGY
312. Olav Magnus Søndenå Fredheim: HEALTH RELATED QUALITY OF LIFE ASSESSMENT AND ASPECTS OF THE CLINICAL PHARMACOLOGY OF METHADONE IN PATIENTS WITH CHRONIC NON-MALIGNANT PAIN
313. Anne Brantberg: FETAL AND PERINATAL IMPLICATIONS OF ANOMALIES IN THE GASTROINTESTINAL TRACT AND THE ABDOMINAL WALL
314. Erik Solligård: GUT LUMINAL MICRODIALYSIS
315. Elin Tollefsen: RESPIRATORY SYMPTOMS IN A COMPREHENSIVE POPULATION BASED STUDY AMONG ADOLESCENTS 13-19 YEARS. YOUNG-HUNT 1995-97 AND 2000-01; THE NORD-TRØNDELAG HEALTH STUDIES (HUNT)
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317. Heidi Knobel: FATIGUE IN CANCER TREATMENT – ASSESSMENT, COURSE AND ETIOLOGY
318. Torbjørn Dahl: CAROTID ARTERY STENOSIS. DIAGNOSTIC AND THERAPEUTIC ASPECTS
319. Inge-Andre Rasmussen jr.: FUNCTIONAL AND DIFFUSION TENSOR MAGNETIC RESONANCE IMAGING IN NEUROSURGICAL PATIENTS
320. Grete Helen Bratberg: PUBERTAL TIMING – ANTECEDENT TO RISK OR RESILIENCE ? EPIDEMIOLOGICAL STUDIES ON GROWTH, MATURATION AND HEALTH RISK BEHAVIOURS; THE YOUNG HUNT STUDY, NORD-TRØNDELAG, NORWAY
321. Sveinung Sørhaug: THE PULMONARY NEUROENDOCRINE SYSTEM. PHYSIOLOGICAL, PATHOLOGICAL AND TUMOURIGENIC ASPECTS
322. Olav Sande Eftedal: ULTRASONIC DETECTION OF DECOMPRESSION INDUCED VASCULAR MICROBUBBLES
323. Rune Bang Leistad: PAIN, AUTONOMIC ACTIVATION AND MUSCULAR ACTIVITY RELATED TO EXPERIMENTALLY-INDUCED COGNITIVE STRESS IN HEADACHE PATIENTS
324. Svein Brekke: TECHNIQUES FOR ENHANCEMENT OF TEMPORAL RESOLUTION IN THREE-DIMENSIONAL ECHOCARDIOGRAPHY
325. Kristian Bernhard Nilsen: AUTONOMIC ACTIVATION AND MUSCLE ACTIVITY IN RELATION TO MUSCULOSKELETAL PAIN

326. Anne Irene Hagen: HEREDITARY BREAST CANCER IN NORWAY. DETECTION AND PROGNOSIS OF BREAST CANCER IN FAMILIES WITH *BRCA1*GENE MUTATION
327. Ingebjørg S. Juel : INTESTINAL INJURY AND RECOVERY AFTER ISCHEMIA. AN EXPERIMENTAL STUDY ON RESTITUTION OF THE SURFACE EPITHELIUM, INTESTINAL PERMEABILITY, AND RELEASE OF BIOMARKERS FROM THE MUCOSA
328. Runa Heimstad: POST-TERM PREGNANCY
329. Jan Egil Afset: ROLE OF ENTEROPATHOGENIC *ESCHERICHIA COLI* IN CHILDHOOD DIARRHOEA IN NORWAY
330. Bent Håvard Hellum: *IN VITRO* INTERACTIONS BETWEEN MEDICINAL DRUGS AND HERBS ON CYTOCHROME P-450 METABOLISM AND P-GLYCOPROTEIN TRANSPORT
331. Morten André Høydal: CARDIAC DYSFUNCTION AND MAXIMAL OXYGEN UPTAKE MYOCARDIAL ADAPTATION TO ENDURANCE TRAINING

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