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Genetic polymorphisms underlying pain report and treatment response of different intra-articular therapeutic modalities –autologous conditioned serum (ACS), hyaluronic acid (HA) and placebo (PL)- in patients suffering from symptomatic midstage knee osteoarthritis

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1. Introduction

1.1 Objective

The goal for thesis was to evaluate genetic polymorphisms, which are associated with pain perception in human patients suffering from symptomatic knee osteoarthritis (question 1). A second goal of this study was to find out if specific genetic markers that correlate with treatment response in regard to different intra-articular treatment modalities in mid stage knee osteoarthritis (KOA) (question 2).

1.2 Hypothesis

1.2.1 Question 1:

Specific single nucleotide polymorphisms (SNPs) exist that are associated with pain report in patients suffering from symptomatic mid-stage knee osteoarthritis.

1.2.2 Question 2:

Certain SNPs show a correlation with treatment response of three different therapeutic intra-articular injections -autologous conditioned serum (ACS), hyaluronic acid (HA) and Saline as placebo (PL)- in patients suffering from symptomatic mid-stage knee osteoarthritis.

1.3 Osteoarthritis

1.3.1 Epidemiology

Osteoarthritis (OA) is a widespread degenerative articular disease, which can affect many joints –most common ones are knee-, hip-, facet joints and joints in hands, more common the distal interphalangeal joints (DIPJs) (Hunter & Felson, 2006). Known risk factors are trauma, the presence of constant micro trauma in form of sports –especially squatting (Heidari, 2011)- and obesity (Hunter & Felson, 2006) as well as genetics (Thakur, Dawes, & McMahon, 2013) beside other known factors. The following box shows a brief summary of relevant facts concerning epidemiology of OA.

- WHO estimated a worldwide prevalence for symptomatic OA of 9.6% of men and 18.0% of women > 60y in 2015 (WHO, 2015)
- Prevalence of radiographic evidence of knee OA (KOA) in adults with the age of >60 years was 37% in the US; Prevalence of symptomatic KOA in adults >60 years with radiographic indices for KOA was 12% in the US (Dillon, Rasch, Gu, & Hirsch, 2006)
- Prevalence of OA lies around 21.1-22.3% in women and 17.9% in men in Germany in 2010. >50% of affected joints were knee OA for both gender (Fuchs, 2013; Robert-Koch-Institut, 2010). Robert-Koch-Institut (RKI) data has been collected through a non-standardized self-register.
- Risk factors: female gender (Cross et al., 2014), obesity for bilateral KOA (Hunter, 2009) (Heidari, 2011; Hochberg et al., 1995), age (Heidari, 2011), previous knee injuries especially for unilateral KOA 3.86 fold (Blagojevic, Jinks, Jeffery, & Jordan, 2010), genetic factors[1](WHO, 2013), estrogen deficiency (Sun, Sturmer, Gunther, & Brenner, 1997)[2], low intake of Vitamin C and D questionable
- Most affected joint being knee joint with consequence of rising global burden to treat KOA (Cross et al., 2014)

1.3.2 Pathophysiology

Furthermore instability, misaligned axis, dysfunction of congruence present predispositions for osteoarthritis (Eaton, 2004; Heidari, 2011). Osteoarthritis occurs when our protective cartilage tissue is worn down and subchondral bone is exposed (Hunter, 2009; WHO, 2013).

It can be said that osteoarthritis manifests especially in load-bearing joints –where a great strength of contraction of periarticular muscles is shown-, rather than the weight-bearing joints (Kenneth D. Brandt, 2010). This may give an attempt to explain why DIPJs are infested amongst other typical joints like knee and hip joint. (Brandt KD, 2010). Osteoarthritis is a complex multifactorial disease with a strong inflammatory component where many components interact (de Rezende, de Campos, & Pailo, 2013; Man & Mologhianu, 2014; Pelletier, Martel-Pelletier, &

[1] Example for genetic factors influencing OA: point mutation 519 from arginine to cysteine in the fibrillar alpha II chain. Genetic mutations causing changes in matric of articular cartilage (Arita M., 2002)

[2] the progression of KOA accelerates after the age of 50 in women, suggesting that lower levels of estrogen may have an effect upon progression in KOA (Kenneth D. Brandt, 2010)

Abramson, 2001; Robinson et al., 2016). Articular cartilage proteolytic degradation occurs due to differed homeostasis in chondrocytes in favor of pro inflammatory cytokines and matrix metalloproteinases (MMPs) and reduced synthesis of collagen type 2 leading to an initial increment in thickness of cartilage, followed by clefts, tears and wearing off (Man & Mologhianu, 2014). Other components in the interplay are metabolism and hypomineralization of subchondral bone, as well as a hyperplastic synovia with presence of proinflammatory cells, such as lymphocytes and synoviocytes (macrophage-like cells) releasing proinflammatory cytokines (like IL-1β, IL-6, TNFα), angiogenic factors (VEGF) and MMPs leading to further inflammation and degradation. It is being discussed whether the synovitis is a consequence or part of pathogenesis of OA. Meniscal damage also plays a role in the pathogenesis. Overall, a variety of joint tissue and a interaction between mechanical and biological factors are suggested to be involved in the underlying pathology of OA (Man & Mologhianu, 2014).

The state of a chronic low-grade inflammation results in peripheral sensitization as well as in central sensitization on spinal level as well as in the brain stem, hypothalamus and cortex. Peripheral afferent endings ‘Silent-nociceptors’ (type III and IV nerve fibres) are activated by inflammation or tissue-damaging stimuli (Grigg, Schaible, & Schmidt, 1986) (Schaible & Schmidt, 1985, 1988). Mechanisms, such as PGE activation and long-term gene induction of nerve growth factor (NGF), tumor necrotic factor (TNF), lower the excitatory level of nociceptors, hence lowering the pain threshold (Kidd, 2012; Kidd, Photiou, & Inglis, 2004). Via neuronal activation of TrkA receptors, NGF causes a sensitization of nociceptive articular afferences, reducing threshold for pain (Kidd, 2012). An amplified response –through NMDA-receptor activation and proinflammatory gene induction-, an increment in receptive field and a modulation of descending inhibitory pathways are mechanisms of central sensitization at spinal level (Kidd et al., 2004)

- Degenerative progress with a low-grade inflammation being a key feature (Heidari, 2011; Robinson et al., 2016)
- Load-bearing joints are especially afflicted (DIPJs) (Kenneth D. Brandt, 2010)
- Initial thickening of cartilage due to increased water content as a consequence of a damaged collagen network. With progression of KOA cartilage thins down due to a reduced production of proteoglycans, leading to vertical clefts. When fibrillated clefts and horizontal tears join, cartilage is worn down and subchondral bone becomes exposed (Heidari, 2011; Kenneth D. Brandt, 2010; Man & Mologhianu, 2014).
- Multiple complex mechanisms lead to peripheral nociceptive sensitization and central sensitization (Grigg et al., 1986; Kidd, 2012; Kidd et al., 2004)

1.3.3 Clinical features and Diagnostics

Symptomatic OA is defined by the attendance of chronic pain and functional disability (Thakur et al., 2013). The major symptom in KOA is joint pain, which aggravates under weight-bearing movement during and after (Hunter & Felson, 2006). Along to the slowly progressive pain are a loss of function so that the range of movement within this joint may be reduced, tenderness or stiffness –especially in the mornings-(Hunter & Felson, 2006; WHO, 2013). A periarticular muscular arthropathy due to pain-related immobility, as well as swelling and increased warmth above the affected joint can be signs of KOA (Brandt KD, 2010). Fever, anemia and weight loss are not initial symptoms in KOA and would rather point towards a rheumatoid problem (Hunter & Felson, 2006). Blood tests and joint fluid analysis can be used to exclude other causes for articular pain, such as local infections or rheumatoid arthritis. A proper anamnestic talk and clinical examination filtering for symptoms described above can enable a doctor to point towards a diagnose KOA. A questionnaire named painDETECT can be used to evaluate whether a neuropathic component exists within the pain disorder of patients with chronic pain (Freynhagen, Baron, Gockel, & Tolle, 2006).

Diagnostic tools

- Detailed anamnesis, including characteristics of pain, medical history, family history, comorbidities such as metabolic diseases, rheumatic diseases
- Questionnaires- eg. WOMAC, painDETECT- used to standardize subjective pain report
- Physical examination looking for swelling, deformation, axial alignment, pain evoking pressure points, restrictions in range of movement (RoM) of a joint, Heberden´s and/or Bouchard nodes, examination of the hip –since diseases affecting the hip can cause knee pain as well-, neurological status –to exclude radiculopathies-
- Sonographic imaging: popliteal Baker cyst, synovia, edema periarticular
- Blood test: inflammatory signs, CRP, IL6, Leukocytes, Rheuma factors, anti-CCP-Antibody
- Synovial fluid analysis: inflammatory signs by evaluating appearance, viscosity, cell count, concentration of glucose.
- Radiological imaging, see below

1.3.4 Radiological Criteria of KOA

Criteria to diagnose Osteoarthritis are joint space narrowing, osteophytes, subchondral sclerosis and subchondral cysts, which can be observed on X-ray-imaging (Kellgren JH, 1957). Above mentioned Symptoms of osteoarthritis often develop gradually over time. Other signs of osteoarthritis may be a grating sensation under pressure due to joint space narrowing or being able to manually feel osteophytes (http://www.mayoclinic.org/diseases-conditions/osteoarthritis/basics/symptoms/con-20014749).

X-ray imaging is the standard diagnostic procedure following clinical examination (WHO, 2013). The Kellgren-Lawrence-Scale can be used to classify osteoarthritis into four grades –criteria being joint space narrowing, osteophytes, subchondral sclerosis and subchondral cysts (J. H. Kellgren, 1957). MRI is used in more complex circumstances and does not count as a standard procedure.

It is well known that a disassociation between radiographic presence of KOA and pain report exists (Hunter & Felson, 2006; Lethbridge-Cejku et al., 1995).

Grade	*Severity of KOA*	*radiographic findings*
0	None	No features of OA
I	Doubtful	Minute osteophyte, doubtful significance
II	Minimal	definite osteophyte, unimpaired joint space
III	Moderate	Moderate diminution of joint space
IV	Severe	Joint space greatly impaired, with sclerosis of subchondral bone

Table 1: Kellgren-Lawrence-Classification scales radiographic structural damage of OA and allows a standardized assessment of the severity of KOA on x-rays. (J. H. Kellgren, 1957)

1.3.5 Therapeutic overview

A step-up approach of therapeutic opportunities, beginning with conservative possibilities, over interventional options to surgical options, is currently state of the art.

Conservative therapy includes pain medication with Acetaminophen, NSAIDs against the inflammatory component (see review), physical therapy to control overweight, orthopedic-technical support such as shoe inserts or psychological therapy to control chronic pain (Wehling P., 2016). Reducing weight (kg) in obese patients plays a center role in non-pharmacological therapeutic strategies due to a correlation between high levels of leptin and risk of KOA (Christensen, Bartels, Astrup, & Bliddal, 2007; Dumond et al., 2003); further details of underlying pathologies of the association is outlined in chapter 2.4.

Interventional therapies are little invasive options for treatment. Local cortisone injections can be applied into the joint to reduce inflammation and pain if NSAIDs and oral pain medication fail to achieve a reasonable pain reduction. Hyaluronic acid is a natural component of joint fluid and is allowed as treatment for osteoarthritis, functioning as a lubricant when also injected intra-articularly. Arthroscopic cartilage-surgery such as abrasio and drilling follow the principle of refreshing the cartilage via micro trauma. A more complex possibility is the mosaic procedure, where a small degenerative area is punched out and replaced with healthy autologous bone-

cartilage tissue from immediate surroundings within the treated joint, which are not exposed to high pressure (http://praxisklinik-heinsberg.de/wordpress/op-infos/arthroskopie-des-kniegelenks/knorpelchirurgie). Interventional modalities include intra-articular injections as well as arthroscopic cartilage-surgery with the goals of reducing inflammation, achieving an analgesic effect and slowing down progression of OA.

A surgical alternative if conservative and interventional therapeutic approaches have failed is arthroplasty, joint replacement surgery with either metal or plastic substances, most commonly done for hip- and kneeOA. Disadvantages to this are that with an invasive procedure the risk of developing postoperative complications such as infection and loosening of the implanted the prosthesis, blood clots followed by thromboembolic incidences, vascular- and nerve injury and other general postoperative complications increases. Challenging is also a follow up surgery if the artificial joint is outworn or needs replacement. Complications may lead to arthrodesis as the ultimo ratio.

conservative approach	surgical/operative approach	intra-articular approach
• Nonpharmacological: education, Moderate Physical exercise, Weight loss in case of obesity • Biomechanical: Thermal modalities: acute pain → cold; chronic pain → warm, Wedged insoles in case of genu vara/valga, Elastic bandages, immobilization splints, Acupuncture • Pharmacological: Analgesics (Acetaminophen, Opioids) NSAIDs, disease-modifying drugs	• Arthroscopic: lavage, debridement, micro fracturing, mosaic techniques • Unicompartimental knee arthroplasty • Total joint arthroplasty • arthrodesis	• 8 FDA-approved Corticosteroids • 7 FDA-approved Hyaluronates • various analgesics • TNFα-Antagonists (Infliximab, Etanercept, Adalimumab) with an intra-joint half-live of 2-4h • IL1-Antagonists Anakinra[3], • Blood-derived: PRP, ACS • Stem cell therapy

Data derived from Wehling et al (Wehling P., 2016). For a detailed overview including brief description & indication please see review on “How does surgery compare with advanced intra-articular therapies in KOA: current thoughts”. The table is supposed to give a brief overview on the great spectrum of possible treatment options.

[3] An indication for TNFα-antagonist as well as for Anakinra is the posttraumatic prevention of KOA –eg after rupture of the anterior cruciate ligament- (Kraus et al., 2012).

1.3.6 Intra-articular therapeutic modalities in knee osteoarthritis

One of the advantages of intra-articular injection is the improvement of directly delivering a drug to the cartilage(Evans, Kraus, & Setton, 2014). Drug efficacy of reaching cartilage is greater than when a drug is applied systemically; a systemically administered drug (eg. p.o. or i.v.) can then only reach cartilage over the subchondral vasculation and afterwards diffusion from synovial fluid into cartilage.

Interventional modalities include intra-articular injections of a variety of substances, such as cortisone, acting anti-inflammatory, and HA, functioning as a natural lubricant. However, there are controversial opinions, meta-analyses and guidelines (example given OARSI guideline 2014 (McAlindon et al., 2014), AAOS recommendation 2013 (Brown, 2013)) on how effective intra-articular injections with hyaluronic acid are. For an extended review on intra-articular review see Evans et al (Evans et al., 2014). Further, systemic side effects are eluded when a drug is applied intra-articular.

A difficulty that comes up in intra-articular therapies is the short dwell time of drugs within a joint, which is a problem in chronic pain conditions. The exit of a drug depends upon its molecular size; micro molecular structures exit via capillaries, whereas macromolecules removal takes place via a rapid lymphatic drainage. Intra-articular half-live of injected drugs alter between minutes and many hours, eg. soluble steroids half-time is between 1-4 hours and HA 12-24 h (Evans et al., 2014).

A newer developed treatment against osteoarthritis is an autologous conditioned serum (ACS), which is injected into the joint after having been processed (Alvarez-Camino, Vazquez-Delgado, & Gay-Escoda, 2013; Baltzer M.D., Moser M.D., Jansen M.D., & Krauspe M.D., 2009; Wehling et al., 2007). ACS is an endogenous cell-free product and hence provides a patient with lower risk of side effects, like allergy, in comparison to reaction against other exogenous substances used in treatment. A standardized processing of ACS includes the collection of 50 ml Blood from a patient using special syringes containing about 200 chromium sulfate-treated glass beads with a diameter of 2,5mm and a surface area of 21 mm^2 (Orthogen, Düsseldorf, Germany). These beads increase the nonpyrogenic surface area and induce a production of Interleukin-1-receptorantagonists (IL-1Ra), other cytokines and growth factors (Wehling et al., 2007). After having collected blood, samples are incubated at +37°C, centrifuged to separate the serum and then filled into 6-8 portions each containing 2ml (Baltzer M.D. et al., 2009). Samples are

afterwards frozen at -20°C and tested for HIV, Hepatitis B, and Hepatitis C (Baltzer M.D. et al., 2009). In a number of individually set sessions ACS is injected intra-articularly.

In comparison to PRP, ACS is a cell-free product without additives (Wehling et al., 2007). There are a number of possibilities to process PRP (Anitua, Sanchez, Orive, & Andia, 2007; Kon et al., 2010; Patel, Dhillon, Aggarwal, Marwaha, & Jain, 2013), hence the outcome of randomized control trials (RCT) evaluating for PRP's efficacy is inconsistent (Ayhan, Kesmezacar, & Akgun, 2014), unlike for ACS with its standardized processing (Wehling P., 2016). Another mentionable advantage over PRP is that to generate ACS a single blood drawing is enough, since it is possible to store and freeze ACS. To generate PRP a blood withdrawal before each injection is necessary (Wehling P., 2016).

1.4 Genetic polymorphisms and its interaction with pain

A single-nucleotide polymorphism is a variant of a single nucleotide within the genome at a specific position with a considerate existence for each variant in the population (>1%) (http://www.nature.com/scitable/definition/single-nucleotide-polymorphism-snp-295).

In many chronic pain disorders SNPs are known to play a vital role in expression and variation within an illness. SNPs occur when a single nucleotide – adenine (A), thymine (T), cytosine (C), guanine (G) or uracil (U) in RNA- within the genome differs to the paired chromosome or members of the same species. They are associated with the expression of many diseases, however most of the time an interaction of many SNPs causes the manifestation of a disease. Very rarely, a single SNP is the sole cause for a disease, then called mendelian disease[4]. Density and variation of SNPs are affected by ethnicity and geography.

SNPs can occur in any region of the genome, however the distribution is heterogeneous, they occur more often in non-coding regions than in coding-regions and intergenetic regions (Barreiro, Laval, Quach, Patin, & Quintana-Murci, 2008). The effect of a SNP depends on its location. SNPs within a coding region can lead to either synonymous change, meaning the sequence does not alter, or non-

[4] Mendelian Genetic Disorders Martin Alexander Kennedy, University of Otago, Christchurch, New Zealand

synonymous change -missense or nonsense SNP- meaning the amino acid sequence changes and the protein fold may vary. SNPs in non-coding regions can still have an effect upon genetic expression by influencing splicing, transcription factor binding to DNA or the stability of messenger RNA, more detail is given in chapter 6.1.

As briefly mentioned above, SNPs can have a great effect upon the variation of an illness and upon the response of a drug. A special pain panel screens for carefully chosen genes and SNPs, which are known to play a role in key pathways of pain perception. With the help of this panel, results as mentioned below about COMT were collected. More detail will be given in Chapter 2.2 explaining the process of genome analysis.

1.4.1 Genetic determinants of symptomatic OA pain

A lack of correlation between radiographic evidence and pain rating is present in OA (Thakur et al., 2013). Lethbridge et al stated that out of a patient collective of 675 Caucasians only 56% out of a subgroup with a Kellgren-Lawrence ≥ 3 were beset with joint pain (Lethbridge-Cejku et al., 1995).

The search for genetic determinants in pain report of OA has obtained priority. Literature research has shown that certain genes have been identified to correlate with pain in clinically relevant OA:

- SCN9A is a gene encoding for the alpha subunit of voltage gated sodium channel located on nociceptors and is known to be vital for pain-related signaling (https://ghr.nlm.nih.gov/gene/SCN9A). A gain of function of this gene was observed in fibromyalgia (Vargas-Alarcon et al., 2012), small fibre neuropathy (Faber et al., 2012) and primary erythromelalgia (Fischer et al., 2009). Reimann et al showed that the SNP rs6746030 A/G (minor allele being A) leading to non-synonymous change Arg-1150-Trp, coding for arginine-to-tryptophane substitution at codon position 1150 was implicated with an increased pain rating in OA, WOMAC being the assessment tool in a radiographic diagnosed OA cohort n=578 (Reimann et al., 2010).
- TRPV1 is a gene encoding for a ligand-gated ion channel located central and peripheral eg on intra-articular nerve endings (Gavenis et al., 2009) and takes a central role in thermal and mechanical pain perception (Caterina, Rosen, Tominaga, Brake, & Julius, 1999; Christoph et al., 2007). Augmentation of

TRPV1-activity induced through proinflammatory factors was found to act as a central mediator for pain in OA as well as in other chronic pain conditions such as migraine, bone cancer and irritable bowel syndrome (IBS) (Alawi & Keeble, 2010; Szallasi & Blumberg, 1999). Through phosphorylation via PKA- PKC-dependent pathways and dephosphorylation TRPV1 can be sensitized and desensitized peripherally respectively, leading to an altered pain threshold (Moriyama et al., 2005). Proinflammatory mediators such as Prostaglandine E2 (PGE2), Prostaglandine I1 (PGI1) and Bradykinin (BK) can trigger the phosphorylation of TRPV1 and causing an augmented pain perception by lowering the threshold (Moriyama et al., 2005). Not only a qualitative change in TRPV1, but also a quantitative change of peripherally expressed TRPV1 on nociceptors can lead to an amplified pain report. Multiple authors showed that NGF-induced TrkA activation resulted in an enhanced expression of TRPV1 (Fernihough, Gentry, Bevan, & Winter, 2005; Ji, Samad, Jin, Schmoll, & Woolf, 2002; Zhang, Huang, & McNaughton, 2005).

An association between a bundle of SNPs in the TRPV1 gene and pain perception has been found (O'Neill et al., 2012). SNP rs8065080 C/T ,Ile585Val allele, correlates with a reduced sensitivity to cold pain (Lotsch, Fluhr, Neddermayer, Doehring, & Geisslinger, 2009). The homozygote genotype Ile-Ile was found to implicate in a 25% lower risk for symptomatic, painful KOA (O'Neill et al., 2012; Valdes et al., 2011).

- PCSK6 encodes for a paired amino acid converting enzyme 4 (PACE4), which belongs to the group of proprotein convertases. By activating cartilage-degrading enzymes, ADAMTS-4 and ADAMTS-5 (short for a disintegrin and metalloproteinase with thrombospondin motifs), it plays a role in underlying pathomechanisms for developing OA (C. B. Little & Fosang, 2010; Seidah et al., 2008).

  Malfait et al discovered that in a cohort of total n=756 the minor allele G of rs900414 A/G was present with a much greater frequency in the asymptomatic KOA subgroup n= 156 in comparison to the symptomatic KOA subgroup n=600 (Malfait et al., 2012). This indicates that the presence of minor allele variant shields against pain in KOA.

  Miller et al showed that PCSK6 knock out mice did not develop pain-associated movement limitations, as a sign for chronic pain, neither did they show central activation of microglia, in comparison to Wildtype mice after 16-week period post destabilization of their medial meniscus (R.E. Miller, April

2014). Both indicate that diminished expression of PACE4 is a protection against painful OA.

- Scientific literature proves that the SNP rs4680, aka Val108/158Met – methionine substituting valine- found within the Catechol-O-Methyltransferase (COMT)[5] gene leads to three different possible haplotypes, associated with different levels of pain sensitivity, low pain sensitivity, average and high pain sensitivity (Diatchenko et al., 2005). Patients who express the haplotype of high pain sensitivity show lower levels of COMT-activity by circa the factor of four (Lotta et al., 1995), leading to higher levels of catecholamine, like norepinephrine, epinephrine and dopamine. This polymorphism is associated with chronic pain conditions such as fibromyalgia (Gursoy et al., 2003) and temporo-mandibular joint disease (TMD) (Marbach & Levitt, 1976). Based on this, it has been found out that Propranolol, a nonselective β-blocker, can lead to a pain reduction in patients expressing the allele associated with lower levels of COMT expression (Tchivileva et al., 2010, p. 9). COMT was found to not only be involved in chronic pain diseases like fibromyalgia and TMD, but also to be related to OA-pain. Studies showed that the presence of the 158Met allele, resulting in a deduced activity of the enzyme, was linked with a 3-fold greater risk for hip pain in females (Tchivileva et al., 2010; van Meurs et al., 2009).
- Generally speaking, expression studies measuring genes functioning, found elevated expressions of proinflammatory factors such as Interleukin 1 and many other cytokines and proinflammatory factors (Thakur et al., 2013). For a detailed listing of genes and references view Thakur et al.

1.5 Objective

The thesis is based upon a hypothesis-driven study looking at SNPs and genes that are known to be or have a strong likelihood to be involved in pain perception. The intent is to detect non-random associations between SNPs and pain rating and SNPs and treatment response in KOA patients.

The first aim of this thesis was to evaluate SNPs involved in pain perception in order to gain greater insight in the underlying pathophysiology in symptomatic KOA.

[5] COMT is a 271 amino acid large enzyme (https://www.ncbi.nlm.nih.gov/protein/CAG30308.1) degrading and inactivating catecholamines and other neuroactive substances by introducing a methyl group to the catechol structure. (https://www.ncbi.nlm.nih.gov/gene/1312; Tchivileva et al., 2010)

The further clinical use may be to act as a prediction on whether an individual patient diagnosis is associated with an increased or deduced pain report.

Secondly, the goal was to analyze for genetic polymorphisms correlating with the therapeutic response with ACS, HA and PL separately. This could act as the basis for a clinical application with a more patient-centered treatment and forecast. This study was the first report of genetic association in terms of treatment response to three different intra-articular modalities in symptomatic KOA patients.

2. Materials and Methods

2.1 Description of the underlying clinical study

The cohort we have used for our analysis has been collected for a study carried out in Germany by Baltzer et al. Their goal was to prove that treatment with autologous conditioned serum has a greater effect on pain reduction in comparison to HA and PL (Baltzer M.D. et al., 2009). The study results were published in "Osteoarthritis and Cartilage" in 2009. The genetic analysis described here was carried out after the end of the treatments and was approved by the local Ethics committee Medical Faculty of Heinrich-Heine University Düsseldorf, which also approved the trial (study number 1988)[6].

In brief, the treatment study was carried as follows: it was a parallel-group design, randomized, 26-week prospective and double-blinded study –patients and observers were blinded, however doctors injecting not-. Beginning of 2003, Patients, diagnosed with primary knee OA, from five orthopedic centers[7] have been recruited for the study. Volunteers were informed of procedures, risks and alternatives and had to give a written consent. Certain criteria of inclusion had to be fulfilled and are listed below:

1. Patients had to be over the age of 30 years and of Caucasian race
2. They had to be diagnosed with primary knee OA for at least three months before the study began
3. Patients needed to be willing to pause pain medication and other NSAIDs for six months, in order to eliminate this as a possible confounder
4. Their KOA had to be grade 2 or 3 on the Kellgren-Lawrence Scale, which classifies OA into 4 grades[8].
5. Their score on the visual analog scale (VAS) had to be above 50mm, on a scale of 0-100mm, to keep the Standard deviation low during analysis, consequently having a smaller dispersion of data.

[6] see Annex V for the positive ethics votum
[7] involved orthopedic centers: Department of Orthopedics, Heinrich-Heine-University Hospital, Düsseldorf, Germany (recruiting institution); Centre for Molecular Orthopedics, Düsseldorf, Germany (recruiting institution and administration site of the study medication); Dr. Beckmann & Jansen Clinic, Mettmann, Germany (recruiting institution); Dr. E. Harnacke Clinic, Krefeld, Germany (recruiting institution)
[8] See Annex I

6. Previous surgery was accepted, as long as it proceeded the time of first injection by at least three months

Criteria of exclusion were as followed:

1. Patients suffering from any systemic or inflammatory joint disease, suffering from crystalline or neuropathic arthropathy
2. Patients suffering from bone cancer, metastasis or tumor-like lesions in immediate proximity to the treated joint
3. Patients showing any hematologic or abnormal clinical values
4. Pregnant or lactating women
5. Patients abusing drugs
6. Patients having had other intra-articular injections within the last six months before first injection
7. Patients with known allergies against substances used in this trial, saline, HA, ACS
8. Patients with a Kellgren-Lawrence scale grade 4 were not eligible

Applying these inclusion and exclusion criteria 376 informed and volunteering patients initially took part in this study, underwent randomization and received in at least one knee one of the three treatment options –ACS, HA or saline as PL-. It was possible that one individual received treatment in both knees, however data only from the more severe knee and thus primary treated knee has been used for the study. The flowchart below shows each steps of screening and inclusion procedures, as well as detailed numbers of patients for each group at different times. Blood has been collected from patients of all three groups, however only blood samples from those patients within the ACS group underwent the processing of ACS as mentioned in the introductory part of this thesis. All patients had two appointments for three following weeks. Patients within ACS group received one 2ml injection of ACS per appointment, thus six injections in total. Patients within the Saline and HA groups received only one injection per week, a total of three injections, since the ethics committee did not allow a total of six injections of 1%-HA-solution and saline. Within the second appointment of each week within these two groups patients received a local/topical treatment with heparin cream.

In the end, 345 patients completed the 26-week, prospective study.

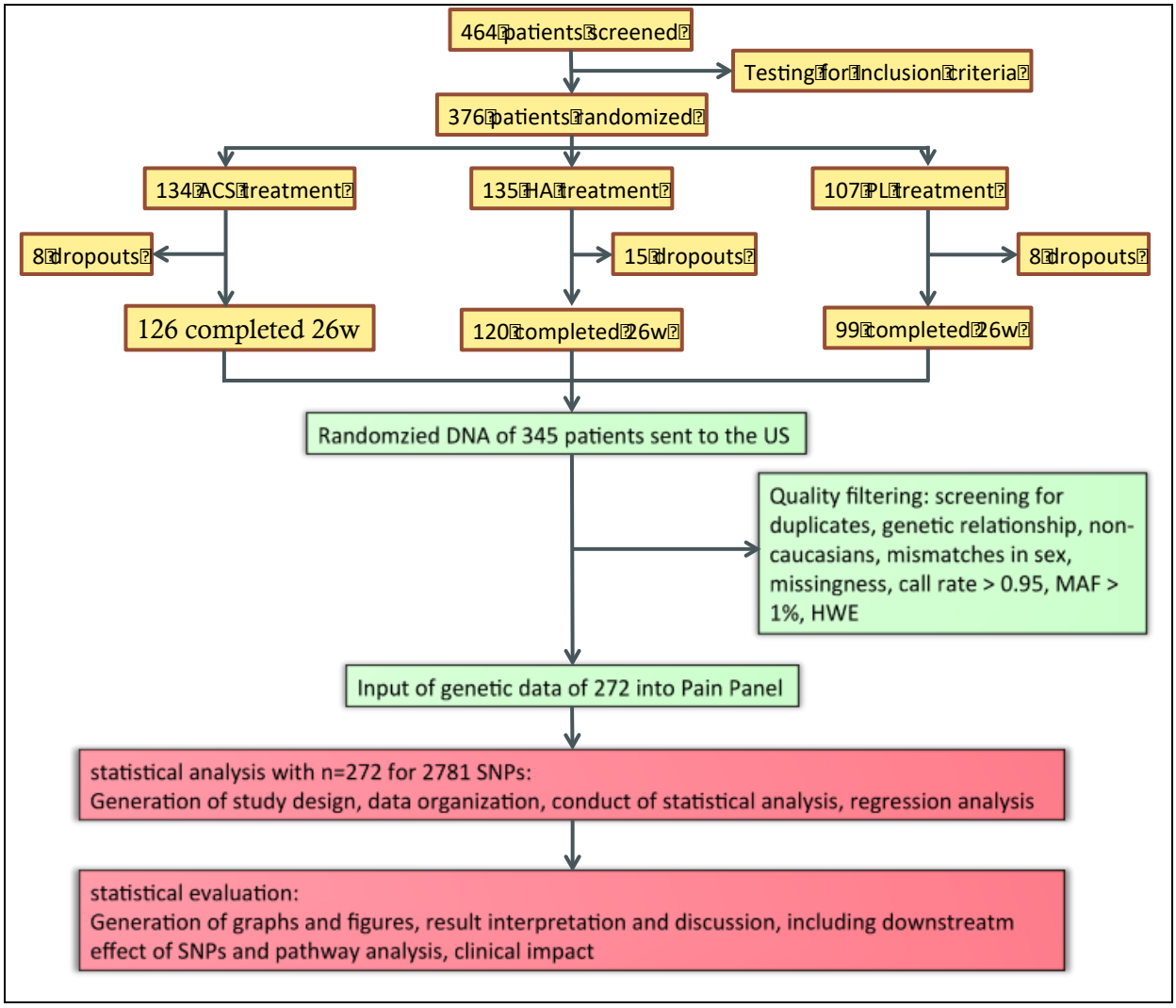


Figure 1: This figure gives a brief overview on how data has been attained. Yellow boxes indicate recruitment of patients and their allocation to one of the treatment arms. For a more detailed description on how data has been collected and reasons for dropouts see review "autologous conditioned serum (ACS) is an effective treatment for knee osteoarthritis" ((Baltzer M.D. et al., 2009). Algynomics and UNC prepared the genomic data set –here represented in green boxes- for statistical analysis, which was the focal point and assignment of this dissertation. The different tasks carried out within the framework of this thesis are listed in red boxes.

Patients had to complete certain Questionnaires visual analog scale (VAS), Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC), Short-Form 8 health-related quality of life survey (SF-8 HRQL) and the global patient assessment of treatment efficacy (GPA) throughout the trial at four different points in time, baseline-before the first injection-, 6 weeks, 3 months and 6 months after their last injection. In our analysis we have used data from VAS, a subjective statement

about the current pain felt with a range from 0-100mm, and the WOMAC[9], which is a disease-specified questionnaire, sub grouped into three categories, pain, physical function and stiffness, made up of 24 questions in total and a range from 0-240mm (Bellamy, Buchanan, Goldsmith, Campbell, & Stitt, 1988).

Parameter		***Subgroups of therapeutic modalities***		
		ACS	***HA***	***PL***
No. injected knees		134	135	107
Average age (y)		53.8 ± 12.2	57.4 ± 12.0	60.3 ± 10.7
Gender (f/m)		65/69	74/61	68/39
History previous knee surgergy (%)		59.4	58.7	60.2
WOMAC (0-10)	***Globalscore (mean)***	5.2 ± 2.3	5.2 ± 2.0	5.2 ± 2.1
	Pain (mean)	5.2 ± 2.4	4.9 ± 2.1	4.9 ± 2.0
	Stifness (mean)	5.6 ± 2.8	6.0 ± 2.7	5.8 ± 2.8
	Function (mean)	5.2 ± 2.4	5.2 ± 2.1	5.2 ± 2.2
VAS (0-100)		69.6 ± 13.1	68.3 ± 12.8	66.3 ± 14.5

Table 2: Display of baseline characteristics of patients -including average age, gender distribution, history of previous knee surgery- and the means with SD of baseline disease attributes of WOMAC and VAS. Clinical data collection and analysis was carried out by Baltzer et al (Baltzer M.D. et al., 2009) stating that statistically indistinguishable for all three subgroups. Analyzing for stratification and correlation between baseline demographics and treatment response showed no significant association (Baltzer M.D. et al., 2009). The table was adapted from Baltzer et al. To clearly state, this data has not been collected by me.

[9] see Annex II

Time points for each subgroups		***Outcome scores***				
			WOMAC			***VAS***
		Global	***Pain***	***Stiffness***	***Function***	
ACS (n=134)	***Baseline***	5.24 ± 2.32	5.18 ± 2.39	5.59 ± 2.70	5.21 ± 2.41	69.6 ± 13.1
	Week 7	2.80 ± 2.30	2.71 ± 2.37	3.07 ± 2.49	2.80 ± 2.34	33.8 ± 23.9
	Week 13	2.42 ± 2.06	2.33 ± 2.14	2.80 ± 2.33	2.40 ± 2.08	29.6 ± 23.1
	Week 26	2.42 ± 2.19	2.42 ± 2.25	2.78 ± 2.45	2.37 ± 2.21	29.5 ± 22.6
HA (n=135)	***Baseline***	5.19 ± 2.04	4.89 ± 2.12	6.04 ± 2.65	5.17 ± 2.11	68.6 ± 12.8
	Week 7	4.02 ± 2.09	3.63 ± 2.09	4.82 ± 2.65	4.04 ± 2.14	52.6 ± 23.2
	Week 13	4.00 ± 2.17	3.73 ± 2.22	4.75 ± 2.68	4.00 ± 2.19	52.1 ± 23.0
	Week 26	3.75 ± 2.42	3.59 ± 2.47	4.32 ± 2.78	3.74 ± 2.44	49.3 ± 25.9
PL (n=107)	***Baseline***	5.16 ± 2.12	4.86 ± 2.01	5.78 ± 2.77	5.18 ± 2.24	66.30 ± 14.5
	Week 7	3.81 ± 2.33	3.49 ± 2.23	4.45 ± 2.89	3.83 ± 2.42	46.70 ± 23.5
	Week 13	3.99 ± 2.13	3.61 ± 2.11	4.69 ± 2.78	4.01 ± 2.20	48.80 ± 22.5
	Week 26	3.93 ± 2.38	3.68 ± 2.24	4.51 ± 2.82	3.94 ± 2.48	48.20 ± 25.6

Table 3: comparison of scores for WOMAC and its subgroups as well as for VAS for each treatment group at all four time points, showing dominance of ACS. (Baltzer M.D. et al., 2009). Baltzer et al have carried out data collection and evaluation beforehand. To clearly state, this data has not been collected by me.

2.2 Technique of genome analysis

Furthermore, DNA has been extracted from blood samples from the 345 participants who completed the 26-week program. This DNA anonymized was transported to America and then underwent the Algynomics Pain Research Panel (http://www.algynomics.com/index.html), a panel designed by Alygnomics[10] screening for 3295 SNPs from 350 genes, which are known to be involved in pain report, hence it is a hypothesis-driven Panel in comparison to GWAS, genome wide association studies which are hypothesis free. Genes from the Algynomics Pain Panel were carefully chosen on their known involvement in nociceptive transmission, inflammation or psychological affects, such as mood (Smith et al., 2012). The Pain Research Panel allows researchers to carry out comprehensive analysis of genetic factors associated with clinical pain. It acts as the connecting key between genomic and phenotypic data and evaluates associations between these. This genetic raw data was the basis of this thesis. Specific and detailed genetic analysis in correlation to pain, function and treatment response were the focus of this work.

2.3 Quality filtering

The evaluated data set needed to undergo a filtering process, to raise its quality and reduce confounders as much as possible. The filtering can be divided into two groups. On the one hand there was filtering, looking at the samples itself. Any duplicates of samples as well as cases showing a genetic relationship between each other, non-Caucasians and mismatches in sex between phenotype and genotype were excluded. Samples undergo a detection filter with their values of probe intensity. Certain boundaries are set and if the SNPs value is outside these boundaries it is said to be a „No Call“. Hence, the call rate represents the SNPs detection. Samples and SNPs with a call rate below 0.95 were excluded.

On the other hand, there is the filter looking at SNPs. Again duplicates, SNPs with a call rate below 0.95 were excluded. If the outcome of a set of data is repeatedly the same, it can be counted as valid and not false. SNPs with repeatability below 98% were excluded, to keep the validation as high as possible.
Minor allele frequency (MAF) refers to the frequency of the less common allele of a sequence variation in a given population. It is used to distinguish between common

[10] under the leadership of William Maixner, DDS, PhD, Luda Diatchenko, MD, PhD, Michael A. Hamilton, Roger B. Fillingim, PhD, Richard H. Gracely, PhD, Gary D. Slade, PhD, Jeffrey S. Mogil, PhD and Alex Sleptsov, PhD, MBA

and rare polymorphisms in a cohort (http://www.ncbi.nlm.nih.gov/projects/SNP/docs/rs_attributes.html). An allele is one of the possible forms a gene or a genetic locus can have caused by polymorphisms. SNPs with MAF below 1% are excluded because when one wants to make a meaningful statement about rare alleles, it requires a strong statistical power.

Hardy-Weinberg-law states that allele or gene frequencies will stay constant from generation to generation: $p^2 + 2pq + q^2 = 1$. Samples and SNPs, which did not follow this equilibrium have been excluded from our set of data.

The process of quality filtering was carried out beforehand by Algynomics and by the University of North Carolina (UNC), as displayed in Figure 1.

2.4 Regression model

Regression tests are statistical models with the aim of detecting a non-random relationship between an outcome variable –dependent variable- and one or more independent variables. We have used regression analysis to identify certain SNPs with a strong predictive power for knee pain in KOA and for treatment response respectively. Statistical outline including chosen variables for our analysis and their general definition are delineated below (please see next page):

Dependent Variable	Question 1: level of pain (VAS or WOMAC) Question 2: treatment response calculated through difference between baseline pain rating and rating at week 26 Therapeutic modalities: ACS -HA -PL	The dependent variable changes in response to a deliberate change of the independent variable
Independent variable	Question 1: presence of SNPs (recessive or dominant SNP) Question 2: presence of SNPs (recessive or dominant SNP)	Change in the independent variable generates a change in dependent variable
Covariates:	Question 1: BMI - age -history of previous surgery Question 2: baseline pain ratings as confounding covariate	A covariate may act as a predictive variable in an analysis, leading to an enhanced estimation of correlation between an independent and dependent variable. Possibilities are: - direct interest - confounding variable: the covariate associates with both dependent and independent variable, in a positive or inverse relation

2.4.1 Idea behind choosing covariates

We have carefully chosen three covariates to evaluate if these are possible factors influencing prediction of the outcome and pain report in KOA patients. The three covariates are age (WHO, 2013) (Hunter & Felson, 2006), history of previous surgery (WHO, 2013) and Body-Mass-Index (BMI)[11] –weight(kg)/height(m)^2-(Hunter & Felson, 2006) (Hunter, 2009) (Hochberg et al., 1995; WHO, 2013). Literature research validates that our covariates are known risk factors in OA.

Higher levels of BMI are accompanied with a larger weight (kg) and may affect the pain report due to greater weight bearing onto the joints. Leptins effect may be the connecting factor between increased risk of OA and rising levels of BMI. Leptin, a 18,64k Dalton protein produced by primarily adipocytes, as well as osteoblasts and chondrocytes may play a key role in KOA (Heidari, 2011). It is hypothesized that Leptin may not only have an influence on the hypothalamus regulating hunger but also on articular cartilage metabolism, activating pro-inflammatory pathways with the risk of a developing OA (Grazio & Balen, 2009; Sandell, 2009; Simopoulou et al., 2007). Higher intra-articular levels of Leptin were observed in human OA (Dumond et al., 2003). Dumond et al provided “evidence that leptin plays a key role in the pathogenesis of OA via stimulation of the synthesis of growth factors and pro-inflammatory mediators” (Dumond et al., 2003).

In literature, an association between history of previous surgery –essentially arthroscopic procedures—and a strong neuropathic pain report, possibly caused through surgical manipulation rather than blunt trauma, was suggested (Valdes, Suokas, Doherty, Jenkins, & Doherty, 2014).

Age may affect pain report in KOA patients. Many cross-sectional epidemiologic studies have shown that the prevalence of chronic pain increases with increasing age (Buskila, Abramov, Biton, & Neumann, 2000; Crook, Rideout, & Browne, 1984). It has to be kept in mind that bias for epidemiological studies on a complex topic like pain sensation exist. Depending on ethnic background, pain sensation may vary, hence results of this dissertation can only be applied to this one ethnicity.

[11] BMI as a characteristics can not only function as a covariate but may also confound our results. Overweight may not only influence pain due to its mechanical force onto the joint, but also through chemical substances released by adipocytes which might modulate pain pathways and may falsify the outcome.

2.5 Software

2.5.1 PLINK

After the data underwent all quality filtering and has been cleaned, 272 samples were left which were then ready to be analyzed using a special software, PLINK. PLINK, developed by Shaun Purcell, is a free-download toolset analyzing genome association of a dataset that is uploaded into the program. PLINK solely focuses on analysis of genotype and phenotype data and does not support any other steps prior to this, such as planning and the study design in general. PLINK is able to carry out a summary statistics for quality control, which gave proof that quality filtering as mentioned above was carried out in a correct manner. PLINKs quality control includes checking for missingness, sex checks and genotype frequencies amongst others (Chang et al., 2015). Furthermore, it is able to carry out basic association tests such as linear regression association studies, which has been mainly used in our analysis of finding SNPs associated with pain report and treatment response (Purcell et al., 2007). Regression tests are statistical models that try to associate an outcome variable with one or more independent variables. We have used this regression analysis to identify certain SNPs with a strong predictive power for knee pain, hence associate genotype with phenotype.

2.5.2 Quantile-Quantile Plots

Population stratification describes an event where allele frequencies differ in subgroups –case and control- within a population, misleading to a false association (Little J, 2009). It is a frequent cause for bias within genetic association studies. Baltzer et al. state in their paper that stratification analyses show no significance.

Quantile-Quantile plots (Q/Q-plot) can be used to represent different statistical tests including a linear regression. The negative logarithm of a p-value is plotted in function of the negative logarithm of a p-value (p), divided by the number of SNPs (n) plus 1:

$$-\log (p/n+1)$$

The x-axis plots the expected –log of p, the y-axis the observed –log of p and each dot on the graph represents one SNP. Derived from this, a Q/Q-plot shows whether the analyzed dataset underlies the Hardy-Weinberg-Equilibrium (HWE) (Little J, 2009). When looking at the left bottom of the graph, SNPs should be in close adherence to the blue line „H0“ (expected in function of expected), which represents

the null hypothesis, then the likelihood of mistakes causing false results is lower and they are likely to follow HWE (Figure 2). Furthermore, SNPs with a very small p-value can be seen on the right top corner of the graph, since the negative logarithm is applied to emphasize a small p-value. The greater the deviation above the blue line, the greater the association between SNP and pain report.

A Q/Q-plot allows detection of “systematic bias due to deviation from HWE, population stratification, genotyping error” (J. Little et al., 2009) by interpreting the curve of the line (Pearson TA, Manolio TA 2008).

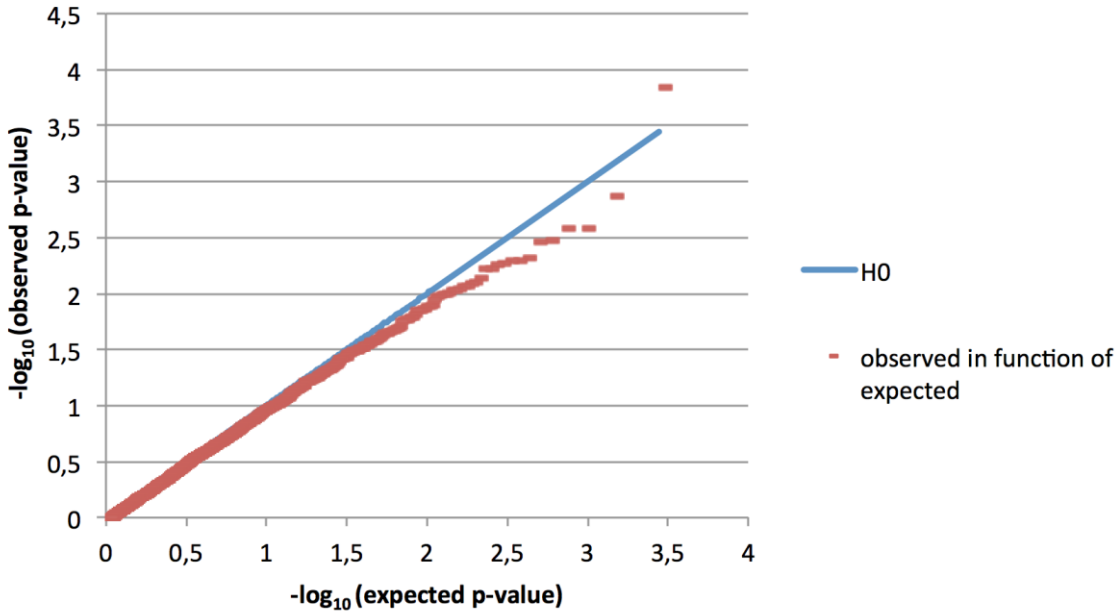


Figure 2: -log Quantile-Quantile plot to illustrate regression test results. The blue line represents the null hypothesis: no association between any SNPs and pain report in KOA patients. Every red dot stands for a p-value for a SNP. Close adherence on the bottom corner tells us there is little false association or systematic bias. One SNP, top right corner above the blue line, shows strong association, since it does not adhere to the blue line. However, it does not show a significant one, since it does not pass the Bonferroni value, which would be at 4,5 of the negative logarithm.

2.5.3 Lambda

When carrying out association analyses, it is critical to test our statistics distribution in comparison with the expected null distribution. This can be done by designing Q/Q-plots (as it has been done above) and by calculating the genomic inflation factor. This factor, lambda (λ), is used to quantify the extent of inflation and false positive rate within a regression analysis. It describes the correlation between an independent and a dependent value. Goal is to achieve a value of λ=1. Values

deviating strongly from 1 may point out undetected confounders such as racial background, unknown familial relationships within our cohort, systemic technical bias or badly calibrated statistics.

2.5.4 Manhattan Plots

Manhattan plots, which can be designed using a program called HaploView, are another possibility to visualize associated SNPs to pain report and where they are located within the genome. The name „Manhattan“ plot is due to its similarity with the Manhattan skyline. It shows genomic coordinates on the x-axis, 23 chromosomes and the negative logarithm of associated p-value of SNPs on the y-axis (Figure 3). The greater the association, the smaller the p-value, hence the larger its negative logarithm.

Each dot represents a SNPs p-value. Using a Manhattan plot it can be outlined which SNP has a strong association and on which chromosome it is located at

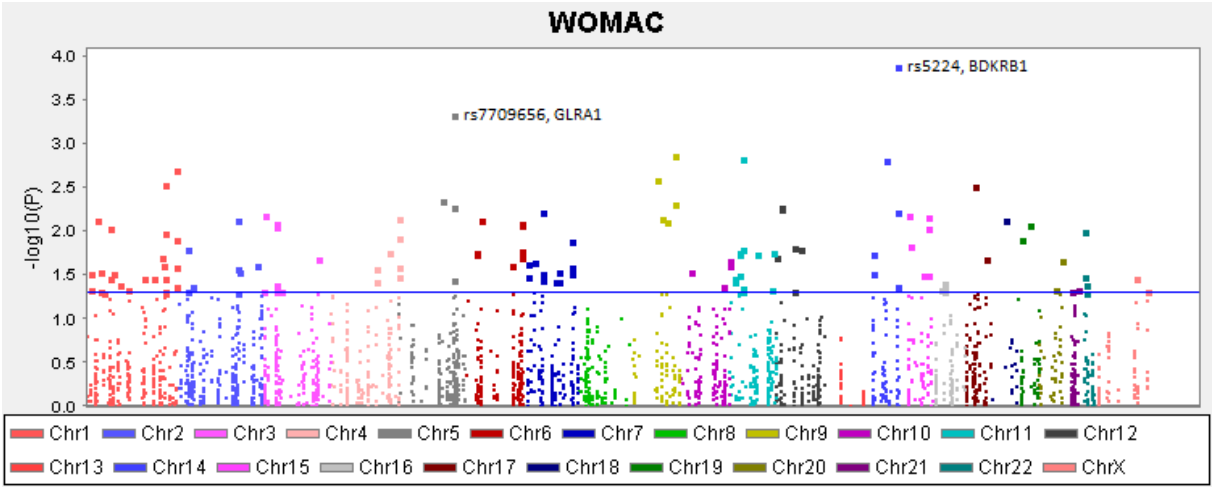


Figure 3: A colour-coordinated x-axis represents haplotypes or SNPs of each region on the genome that were tested, organized chromosome (each dot being one haplotype). The negative logarithm of p-value is displayed on the y-axis, representing the strength of association between the haplotype and the phenotype being measured. The greater an association, the smaller the p-value, hence when taking its negative logarithm, it will be larger and easier to illustrate visually. Each dot illustrates a SNP´s p-value in a regression model. The blue line indicates the gemone-wide significance niveau before the Bonferroni correction, which is explained further on in Chapter 3.1.

2.5.5 Linkage Disequilibrium diagrams

Linkage disequilibrium (LD) describes a non-random relationship of genetic alleles and SNPs within a given population. In statistical analysis LD describes the spurious association between a SNP and a genetic expression. This SNP however only seems to be in a significant association with genetic expression – in our project pain report-, only because it is in strong LD with a SNP that has the actual impact on genetic regulation.

Linkage disequilibrium can be caused due to limited recombination during meiosis within a chromosome; the consequence is that certain SNPs are to be inherited together above average. Certain hotspots of recombination exist and can be diagrammed via LocusZoom, a program, which will be explained in more detail further on in this thesis. Along to this, LD can be caused by mutation or genetic drift- the change in frequency of genetic variations-.

The LD-coefficient r^2 represents the correlation squared between two defined SNPs. It is outlined in a LD-box for two SNPs and can obtain values between $r^2=0$, meaning the two SNPs are not inherited together, and $r^2=100$, implying a complete inheritance of the two SNPs (Figure 4). LD measures the degree to which alleles at two different loci are non-randomly associated (https://www.ndsu.edu/pubweb/~mcclean/plsc731/Linkage%20Disequilibrium%20-%20Association%20Mapping%20in%20Plants-lecture-overheads.pdf).

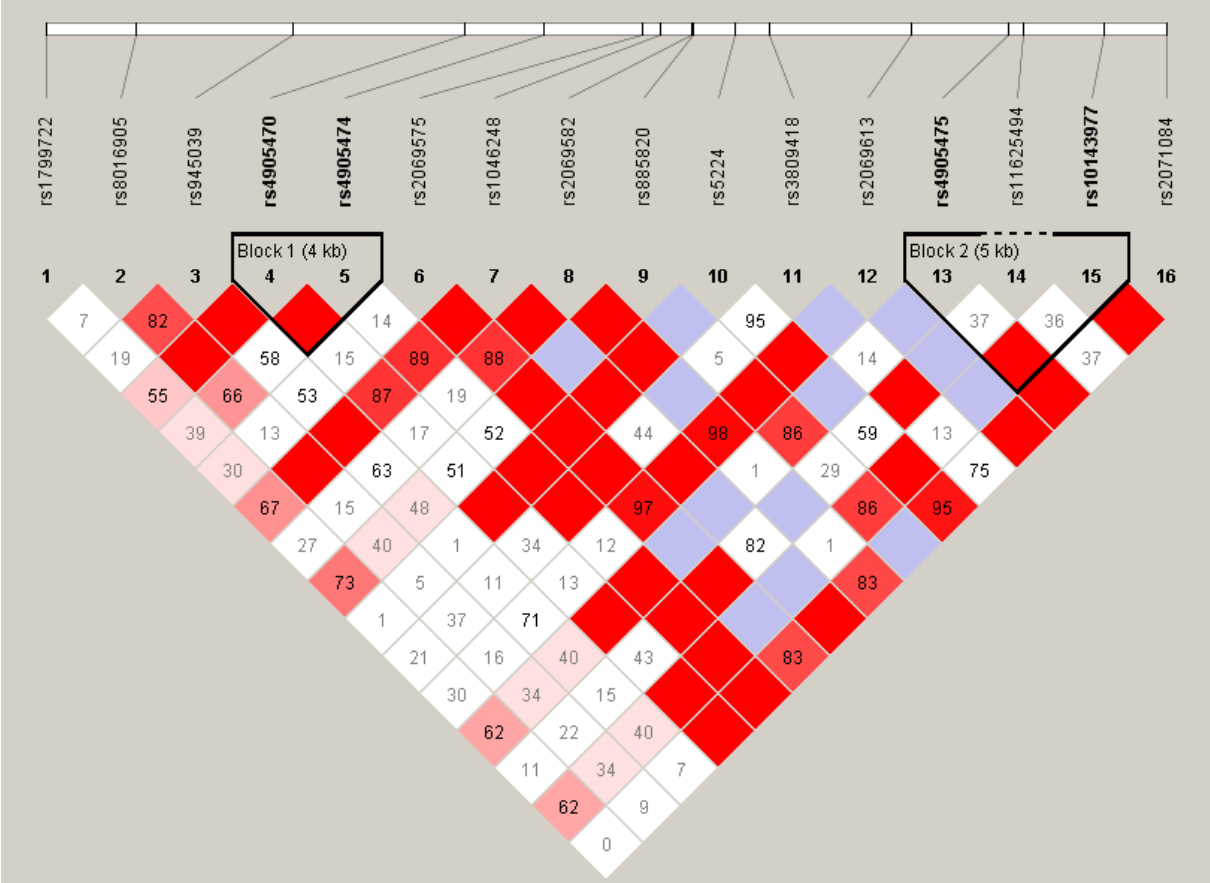


Figure 4: Figure 4 shows a LD-plot with r^2, the LD-coefficients, for pairwise SNPs. Each square with its r^2 represents the strength of a non-random relation between two SNPs. Location of the SNPs within the gene can be obtained on the white bar at the top.

2.5.6 LocusZoom

LocusZoom is a web-based tool developed by Prium et al. with the intention of visualizing GWAS[12] results in an easy and publishable manner. The programs' functions are briefly listed below:

- Visual display of regional information of a SNP in relation to genomic positions and other SNPs
- Linkage disequilibrium and its magnitude to other SNPs → colour-coded points explained in legend in top right corner r^2
- Recombination patterns with hotspots → blue line on which spikes represent known recombination hotspots
- Genes in close position to the region of interest → parallel to x-axis

[12] Genome-wide association studies

This tool enables us to visualize more information of our index SNP as it will be shown along this dissertation. For further information and more detailed explanation see (Pruim et al., 2010).

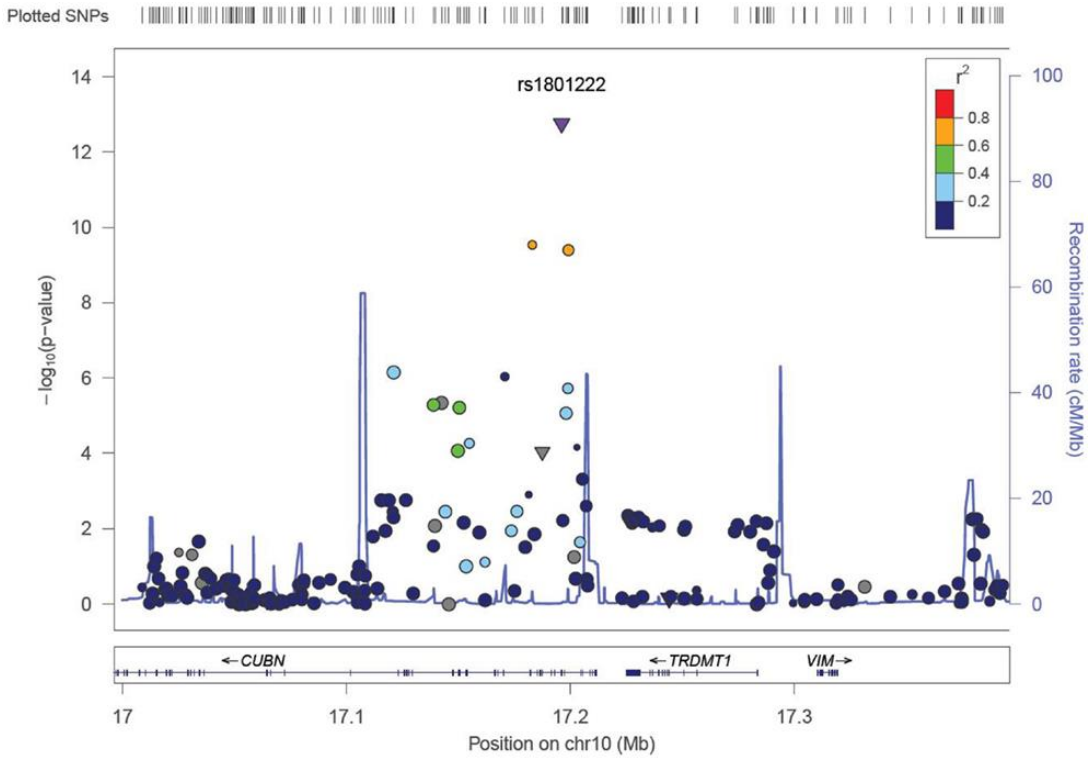


Figure 5: Model LocusZoom for the index SNP.

3. Results

3.1 Question 1: evaluating SNPs associated with pain perception in KOA

Due to the underpowered nature of our study -since there is a relative small sample size (n= 272)- the critical value of significance after the Bonferroni-correction was set to $p < 1.892e^{-5}$. The Bonferroni-correction adjusts the significance threshold for the estimated effective number of independent SNPs, not the total number of SNPs in the Pain Panel due to false association via LD (Smith et al., 2012). Carrying out a linear regression analysis using PLINK four SNPs were found that have gotten close to our significance niveau.

For our analysis we have used the phenotype of VAS and the pain category of the WOMAC at baseline. Hence, two sets of results were obtained for each linear regression test carried out. Results are shown below.

Initial screening and data collection at baseline was carried out by Baltzer et al. Technical quality filtering of DNA was carried out in the US by University of North Carolina (UNC) and Algynomics. Tasks involved in this thesis were:

- Generation of the study design
- Data organization
- Complex conduct of statistical analysis
- Generating graphs and figures
- Result interpretation and detailed discussion of results, downstream effect of SNPs and pathway analysis
- Conclusion-drawing and clinical impact

3.2 Linear regression analysis applying covariates

As mentioned above, regression tests with covariates were also carried out. Our three covariates –age, history of previous surgery and BMI- were applied separately, as well as in combination of two or three covariates within a linear regression analysis.

P-values tell us if an association between genotype/SNP and phenotype/altered pain perception exists. Beta reveals the actual effect of the SNP on altered pain report. If beta is a positive value, the SNP is associated with an

increment of pain report. Whereas a negative value of beta, indicates that the SNP is associated with a deduced pain report.

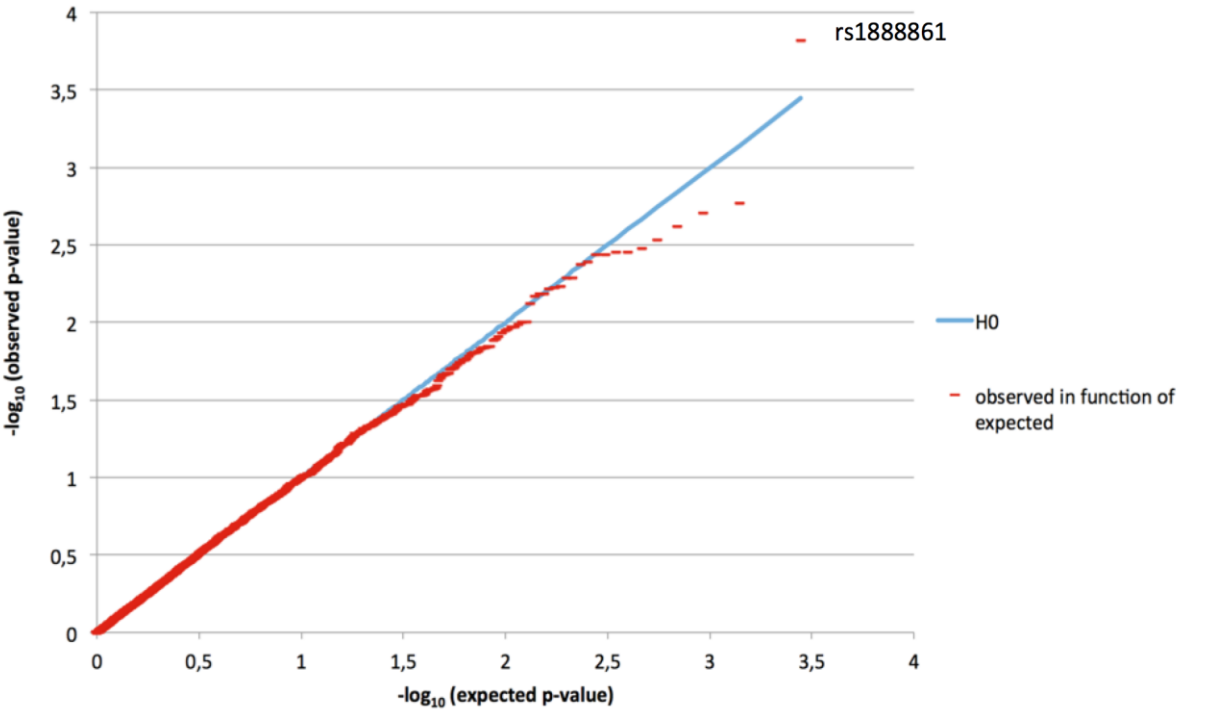
Subchapters below show results for each covariate applied in linear regression analysis separately.

3.2.1 Linear regression applying a combination of all three covariates

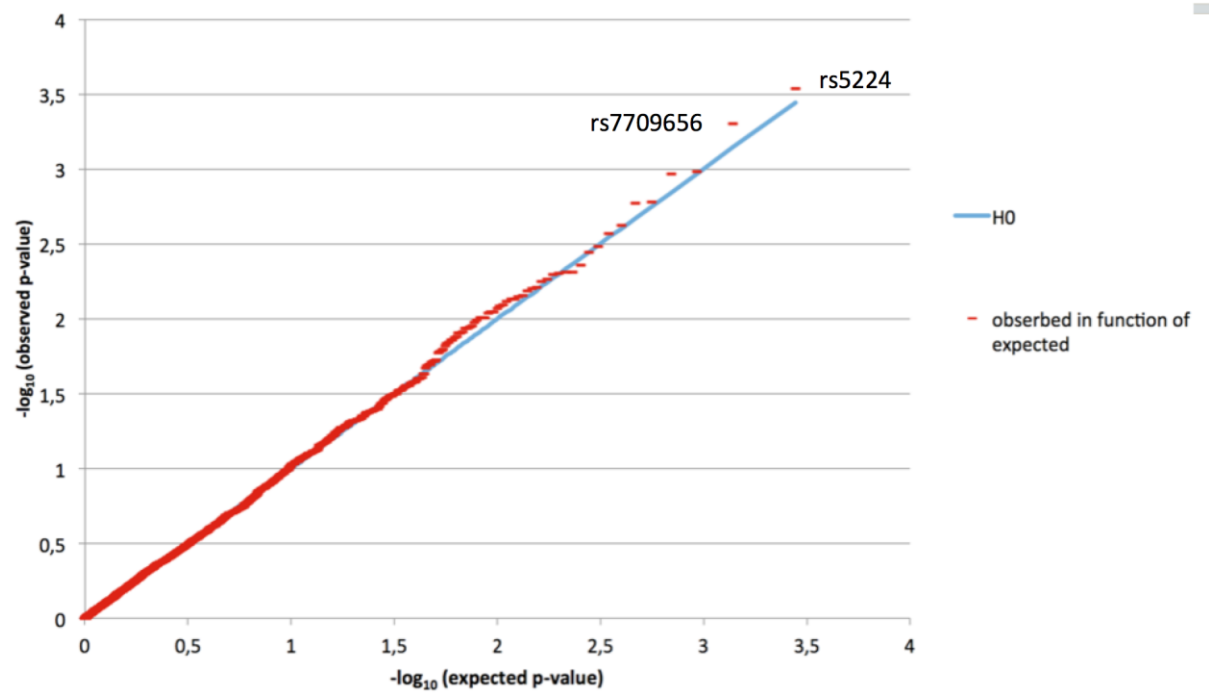
Primary analysis to have been carried out was a regression analysis where the three covariates are all taken into calculation.

SNP	***Gene***	***Chr***	***p-value***	***Beta***
WOMAC				
rs5224	BDKRB2	14	0.0002919	4.749
rs7709656	GLRA1	5	0.0004998	4.728
rs1236913	PTGS1	9	0.00105	6.472
rs554576	CAT	11	0.001084	3.532
VAS				
rs1888861	TrkA	1	0.0001526	-5.010
rs548339	OPRM1	6	0.001716	3.879
rs14138	PRKCE	2	0.001976	5.732

Table 4: This table displays four most significant SNPs when WOMAC was applied and the three most significant SNPs under application of VAS including which gene they belong to and its molecular location (chromosome). rs5224 and rs7709656 come close to the significance threshold and set themselves apart from the following two SNPs with a much less significant p-value. Similar can be observed for rs1888861, delimiting itself with its p-value from the following SNPs. Beta describes the direction and size of the effect on pain perception. Rs5224 and rs7709656 are associated with an increment in pain report in this dataset, whereas rs1888861 is associated with a decreased pain report.



6a. VAS



6b. WOMAC

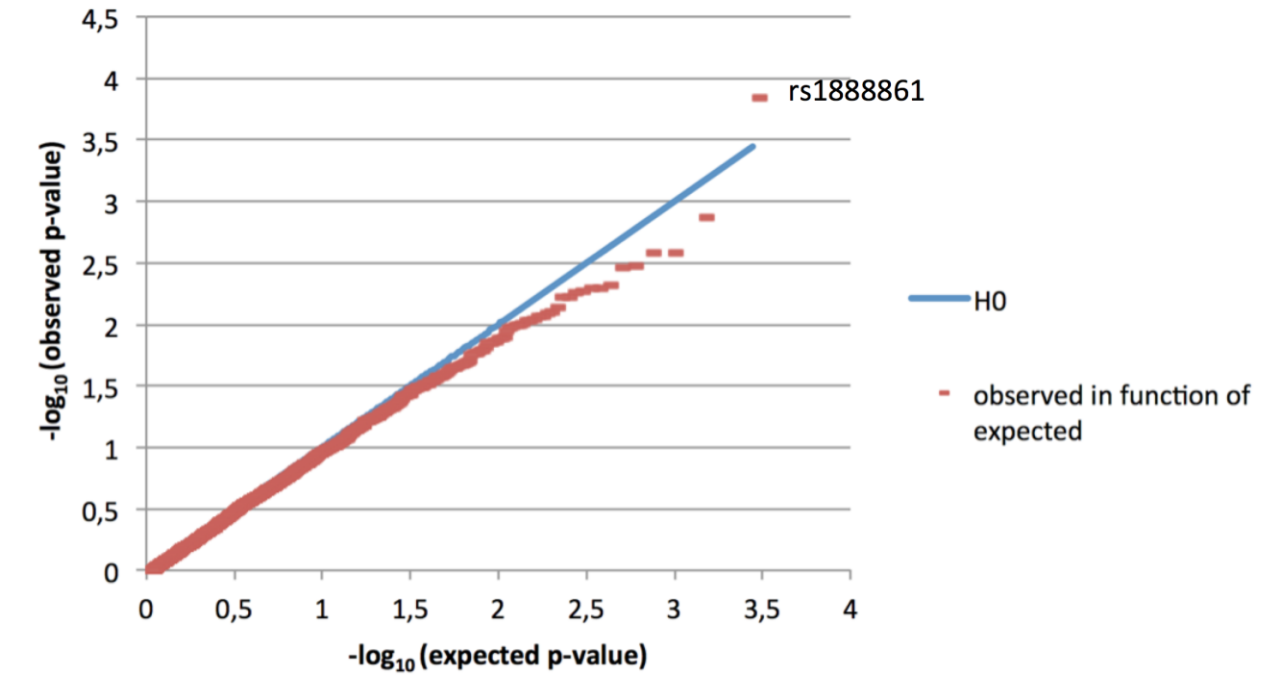
Figure 6: the general shape of both Q/Q-plots (under application of VAS and WOMAC respectively) give reason to believe that systematic bias is absent in this regression analysis. Lambda 1.02. The marked dots represent the SNPs from Table 4 above, which show a non-random association to altered pain report in participants of this study.

To estimate whether associations hold up without a particular covariate, regression analysis was carried out applying each covariate separately. If the association does not hold up, then the absent covariate could be counted as a confounder.

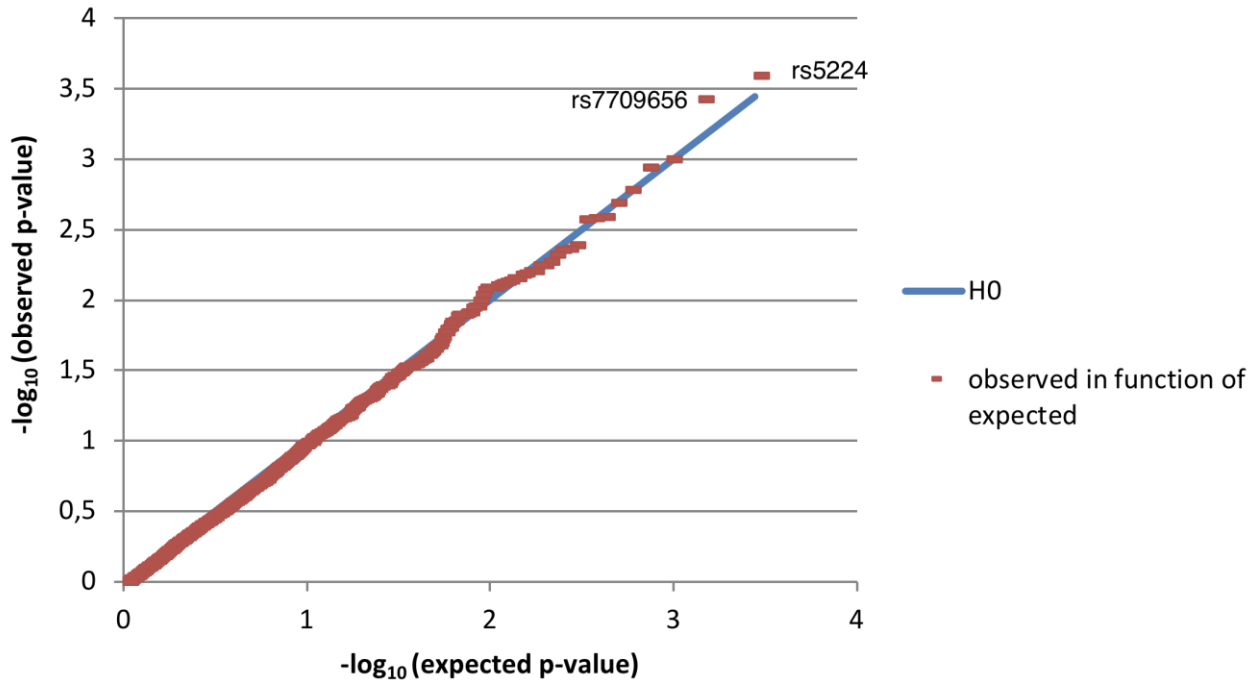
3.2.2. Age as a covariate

SNP	***Gene***	***Chr***	***p-value***	***Beta***
WOMAC				
rs5224	BDKRB2	14	0.0002552	4.779
rs7709656	GLRA1	5	0.0003759	4.799
rs1236913	PTGS1	9	0.0009936	6.494
rs554576	CAT	11	0.001156	3.505
VAS				
rs1888861	TrkA	1	0.0001441	-5.042
rs3758987	HTR3B	11	0.001354	4.497
rs7825588	NRG1	8	0.00153	-0.254

Table 5: Results for linear regression analysis when age is taken into account as a covariate are displayed in this table. Three SNPs in total were found with a stronger association, rs5224 and rs7709656 when WOMAC is used for the phenotypic data and one SNP, rs1888861, when VAS phenotype is used. The other listed SNPs have been added to the table to illustrate the deviation of rs5224, rs7709656 and rs1888861 from the rest.
It can be observed that most significant SNPs for regression analysis when age is applied as a sole covariate do not differ from SNPs in analysis when three covariates are applied.



7a. VAS



7b. WOMAC

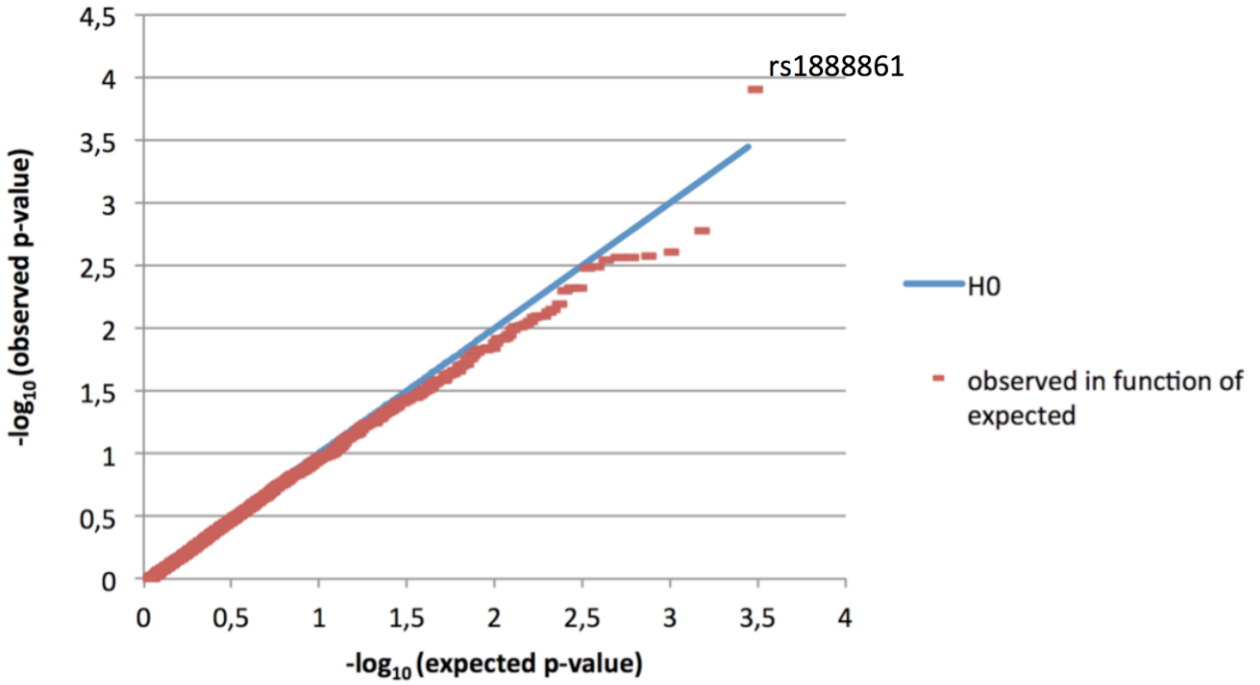
Figure 7: a. Q/Q-plot applying VAS data with age as a covariate. The marked dot deviating from H0 represents the SNP rs1888861. By looking at the shape of the curve showing no general deviation from H0, systematic bias can be ruled out, Lambda 0.99. b. Graph represents data from table 5 using WOMAC data. The marked dots stand for the two most significant SNPs rs5224, rs7709656. Again, systematic bias can be excluded, Lambda 0.99.

The Quantile-Quantile-plots represent the relationship between the SNPs and their association to pain report in KOA. The Blue line illustrates the Null hypothesis: an association between SNPs and pain report in KOA patients does not exist. Dots deviated above the blue line represent our SNPs from table 5, which suggest a great association. Further, the data is not skewed and adheres to the blue line, indicating that association is true.

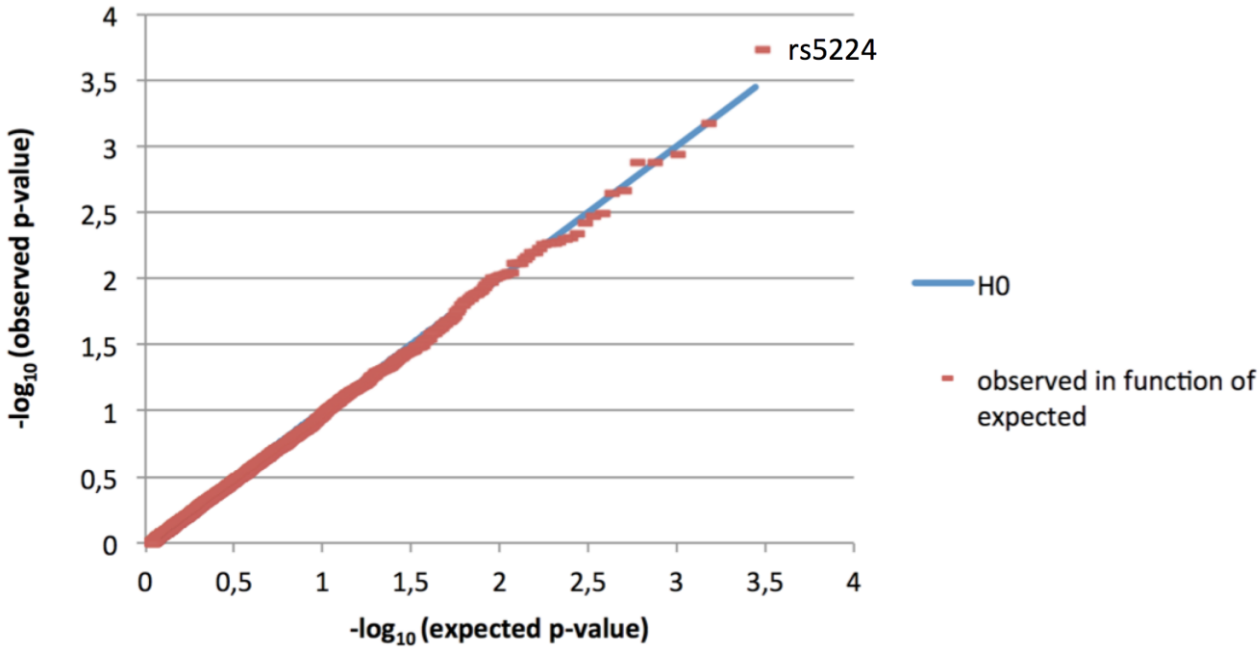
3.2.3 History of previous surgery as a covariate

SNP	***Gene***	***Chr***	***p-value***	***Beta***
WOMAC				
rs5224	BDKRB2	14	0.0001876	4.885
rs7709656	GLRA1	5	0.0006763	4.613
rs554576	CAT	11	0.001152	3.512
rs10137185	ESR2	14	0.001323	-5.958
VAS				
rs1888861	TrkA	1	0.0001264	-5.089
rs548339	OPRM1	6	0.001656	3.330
rs3780446	GABBR2	9	0.002499	-4.246

Table 6: Results for linear regression analysis when history of previous surgery is taken into account as a covariate are displayed in this table. rs5224, when WOMAC is used for the phenotypic data, and rs1888861, when VAS phenotype is applied, showed a strong correlation $p > 0.0002$ to altered pain perception. Association between Rs7709656 and enhanced pain perception is present; the correlation is not as strong as in analysis with all three covariates. In comparison to analysis under application of all three covariates, SNPs with the greatest significance niveau overlap, indicating that history of previous surgery as a covariate does not confound the analysis.



8a. VAS



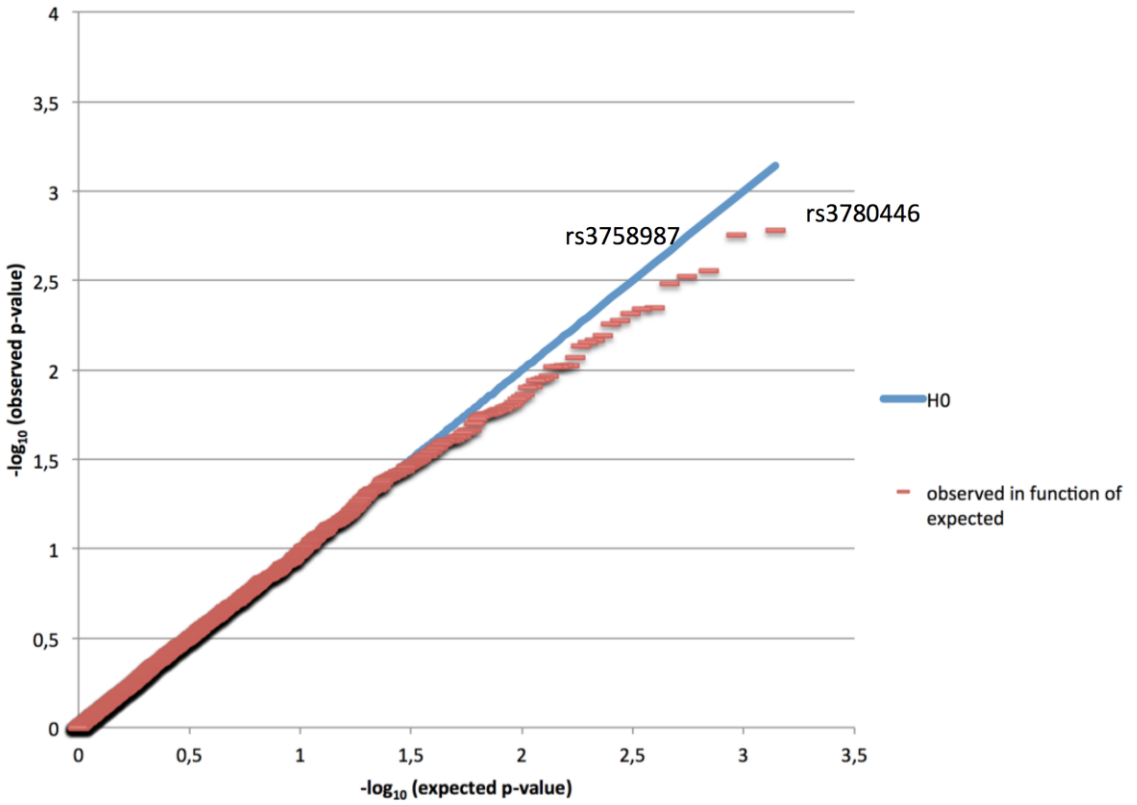
8b. WOMAC

Figure 8: a. and b. show Q/Q-plots applying VAS data and WOMAC data respectively with previous history of surgery as a covariate. The marked dots deviating from H0 represent the SNPs rs1888861 and rs5224 with the strongest association (p = 0.0001264 and p = 0.0001876 respectively). Systematic bias can be ruled out due to a Lambda of 0.99 and the close adherence to H0, except for mentioned SNPs.

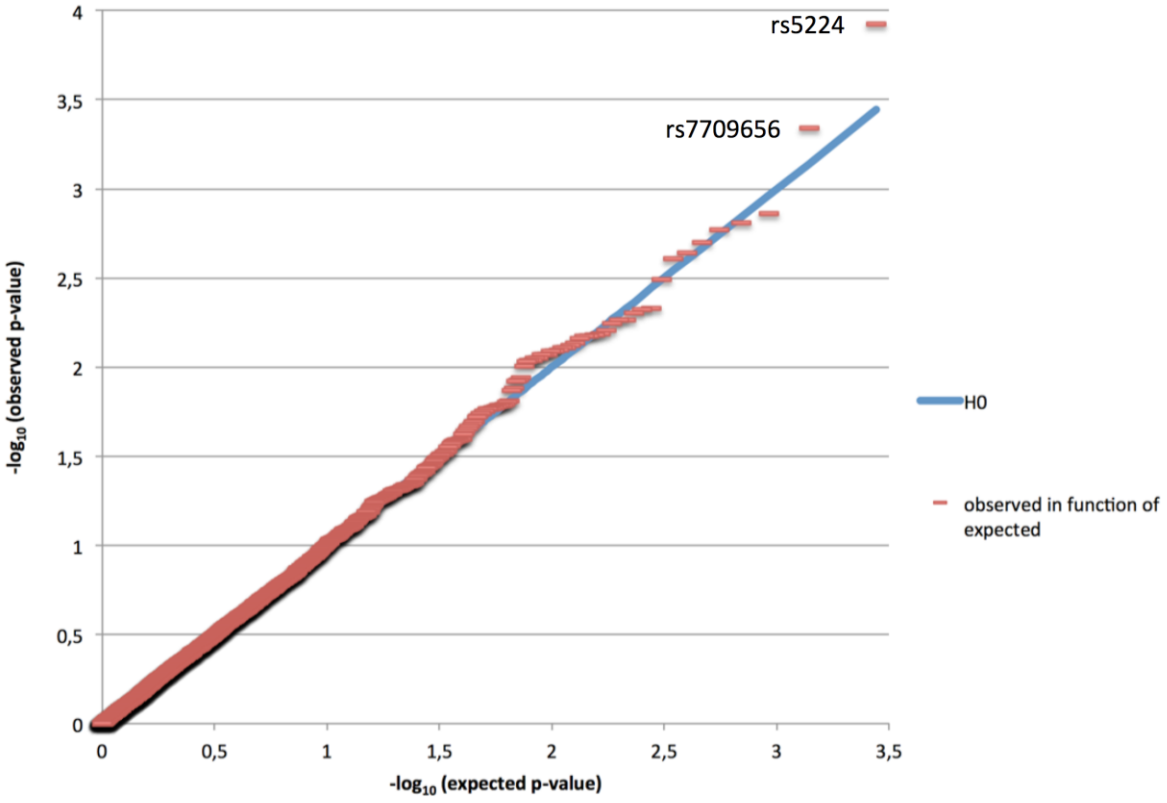
3.2.4 Body-Mass-Index (BMI) as a covariate

SNP	***Gene***	***Chr***	***p-value***	***Beta***
WOMAC				
rs5224	BDKRB2	14	0.0001194	4.999
rs7709656	GLRA1	5	0.0004584	4.751
rs1236913	PTGS1	9	0.001379	6.335
rs554576	CAT	11	0.001548	3.431
VAS				
rs3780446	GABBR2	9	0.001653	-4.457
rs3758987	HTR3B	11	0.00175	4.445
rs1888861	TrkA	1	0.09447	-5.231

Table 7: Results for linear regression analysis when BMI is taken into account as a covariate are displayed in this table. Two SNPs in total were found with a stronger association, rs5224 and rs7709656 when WOMAC is used for phenotypic data. Table displays furthermore the most significant SNP rs3780446 when VAS phenotype is used, however with a low level of p-value. For comparison rs1888861 is listed. Both SNP are well below the significance threshold of $p < 1.892e^{-5}$.



9a. VAS



9b. WOMAC

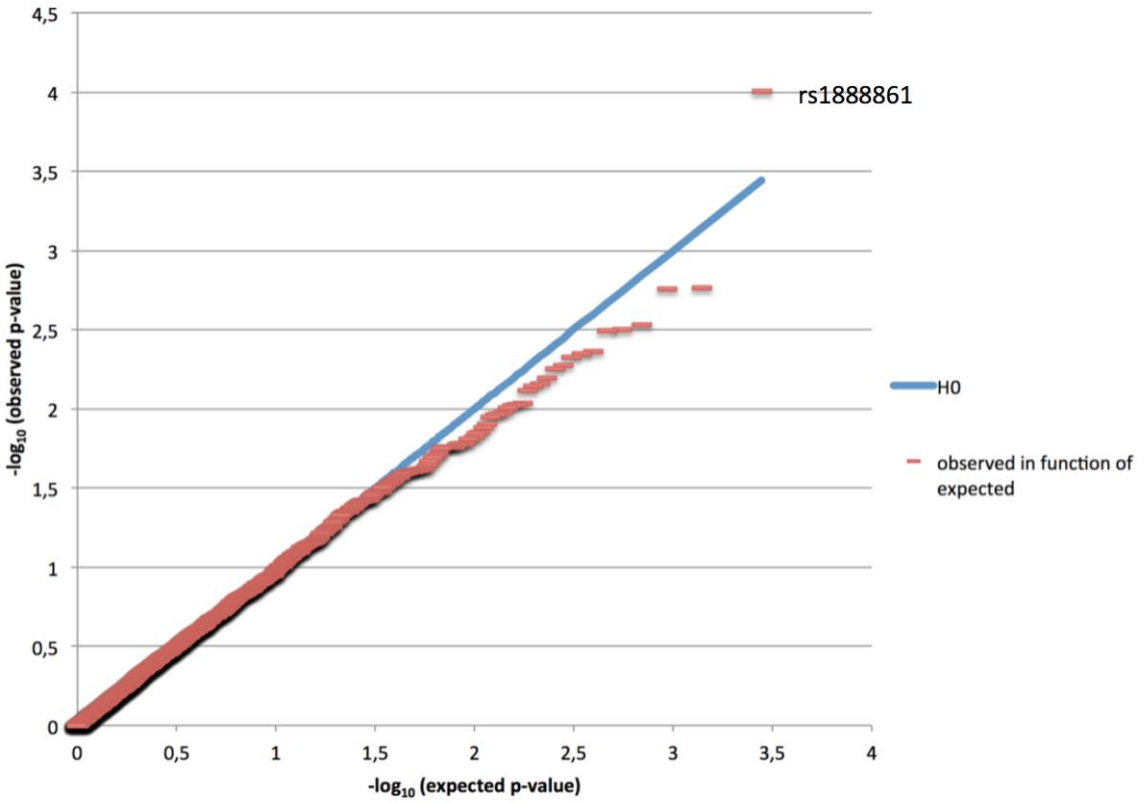
Figure 9: a. VAS. Using phenotypic VAS data, no SNP appear to pass or come close to the significance threshold of $p < 1{,}892e^{-05}$. b. Using WOMAC phenotypic data, SNP rs5224 and rs7709656 are graphed above H0, coming close to the significance threshold. Lambda 1.01

3.3 Linear regression test without application of covariates

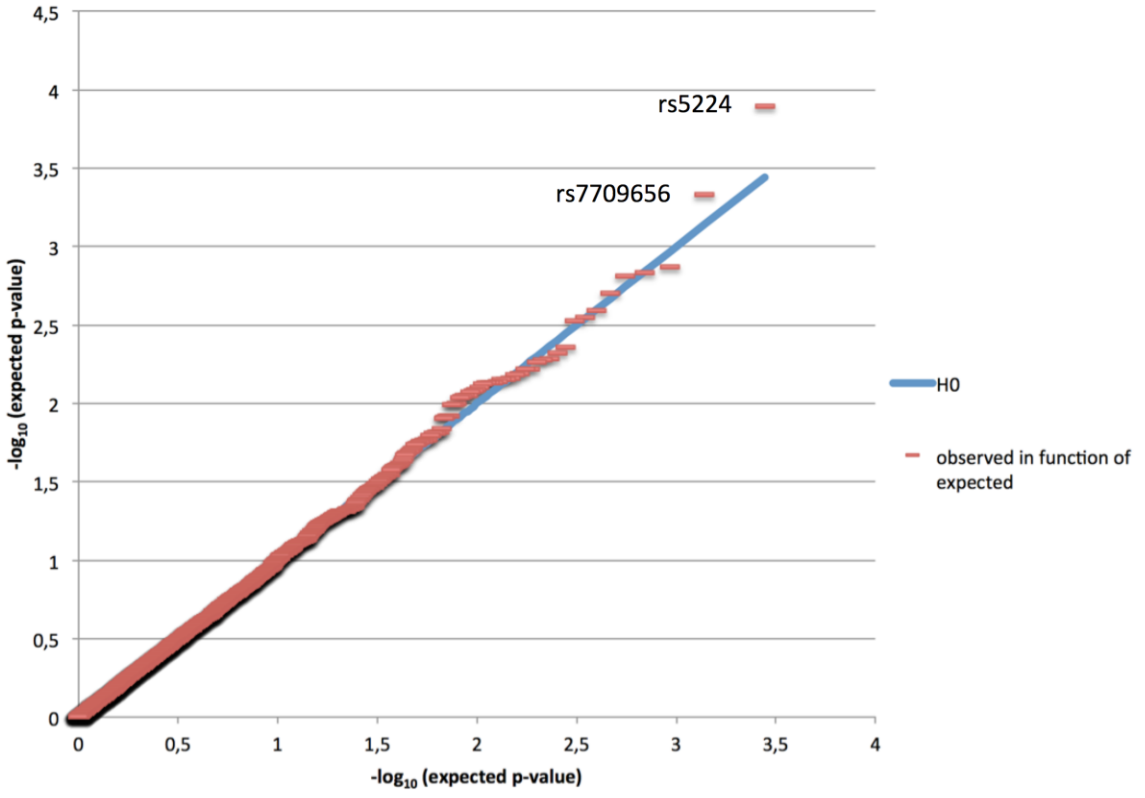
After having found SNPs associated with alteration in pain report upon applying covariates, we looked at the results form regression analysis applying no covariates.

SNP	***Gene***	***Chr***	***p-value***	***Beta***
WOMAC				
rs5224	BDKRB2	14	0.0001269	4.968
rs7709656	GLRA1	5	0.0004655	4.736
rs1236913	PTGS1	9	0.001353	6.335
rs554576	CAT	14	0.001459	3.442
VAS				
rs1888861	TrkA	1	0.0000986	-5.206
rs3758987	HTR3B	11	0.001723	4.444
rs3780446	GABBR2	9	0.001758	-4.420

Table 8: Table displays results of SNPs of the linear regression analysis when no covariates are applied. Again, the known SNPs rs5224, rs7709656 and rs1888861 correlate with a differed pain perception in regression analysis without applying covariates. Rs1888861 comes very close to the significance niveau when no covariates are applied ($p = 9.86e^{-05}$). Similar to above-listed regression studies under application of covariates, p-values of the three SNPs strongly deviate from p-values of the other SNPs tested.



10a. VAS



10b. WOMAC

Figure 10: Similar traits to the other regression analyses can be observed in graphed Q/Q-plots applying no covariates. Rs1888861 as well as rs5224 and rs7709656 for VAS and WOMAC phenotype, respectively, noticeably deviate from H0, indicating an association between these SNPs and alteration in pain report.

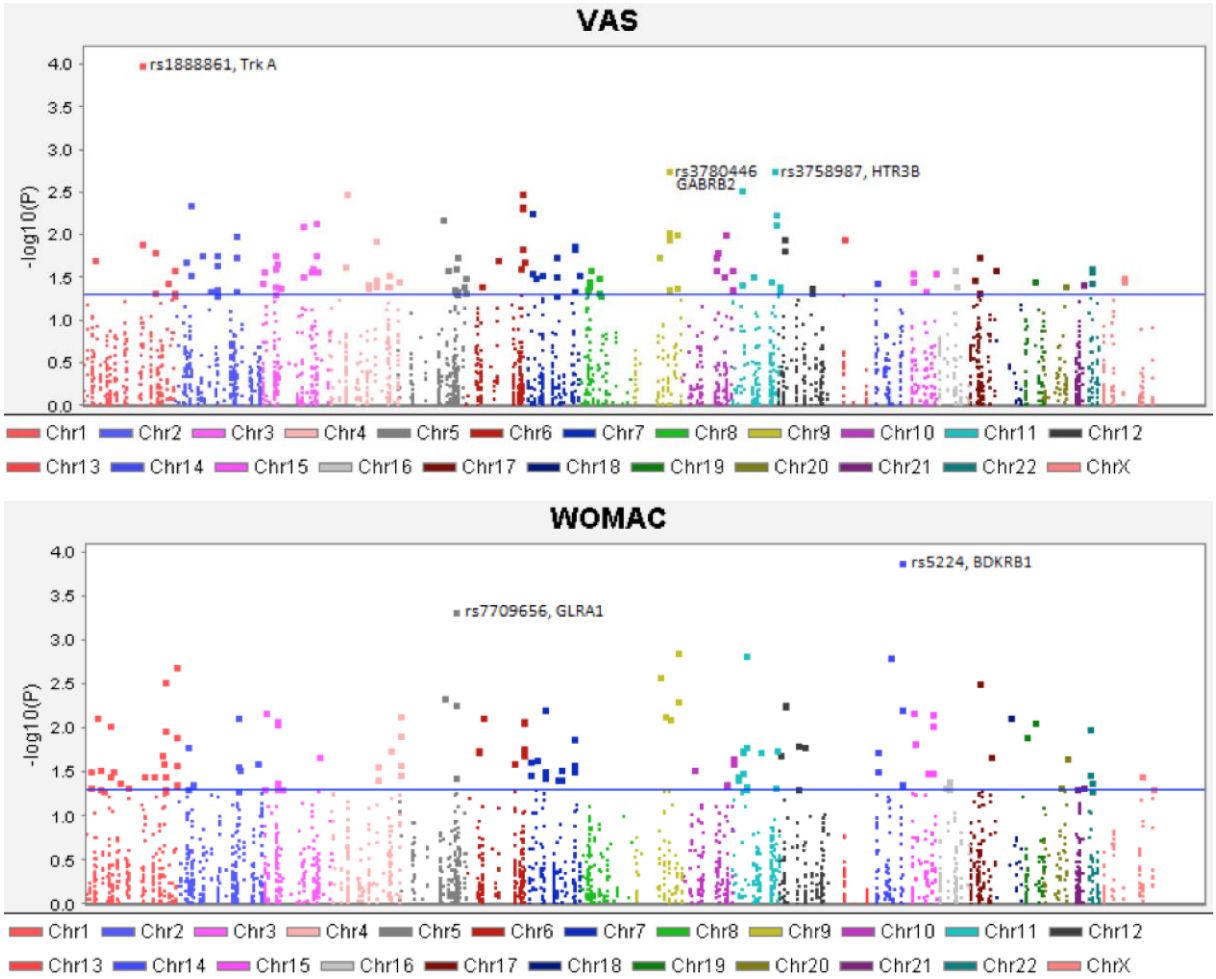


Figure 11: Another possibility to visualize our index SNPs is through a Manhattan plot. SNPs and its belonging genes are marked on both plots for VAS and WOMAC, representing data from table 8. On the top Manhattan plot it can be seen that rs1888861, TrkA located on chromosome 1 is segregated through its large –log of p; whereas when applying WOMAC data it can be seen that rs5224, BDKRB2 located on chromosome 14 and rs7709656, GLRA1 on chromosome 5 are secluded from others implicating possible clinical relevance.

Analysis showed that rs5224 BDKRB2 and rs7709656 GLRA1 appear to be the top SNPs for all regression studies (under application of the phenotypic WOMAC data) with all three covariates applied, age alone, BMI alone and previous history alone. It can be concluded that our covariates do not confound the results when applied in combination or as a sole covariate. Presence of the minor allele of these two SNPs is continuously associated with an amplified pain perception in participants of the study.

Further, regression tests showed that rs1888861 TrkA was constantly the SNP with the strongest association in altered pain report when using VAS as phenotypic

data. This can be said for all regression analyses, except for the analysis with BMI as a sole covariate, here rs1888861 was not associated with differed pain report. The minor allele of Rs1888861 TrkA appears to be associated with a diminished pain report.

See table 9 below for visual depiction of collected information about SNPs.

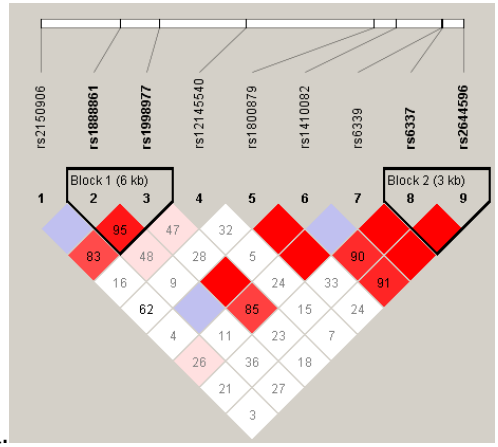
3.4 SNPs & Proteins

SNP	***Gene***	***Chr***	***Location on Chr (bp)***	***Protein***	***Ligand***	***Effect on pain report (beta)***
rs1888861	TrkA	1	155064585	Tropomyosin receptor kinase A	neurotrophin, nerve growth factor (NGF)	↓
rs5224	BDKRB2	14	95777210	Bradykinin receptor B2	Bradykinin, Kallidin	↑
rs7709656	GLRA1	5	151270918	glycin receptor alpha 1	Glycin, β-alanine, Taurine	↑
rs3780446	GABBR2	9	100111383	GABA receptor B2	γ-aminobyturic-acid (GABA)	↓

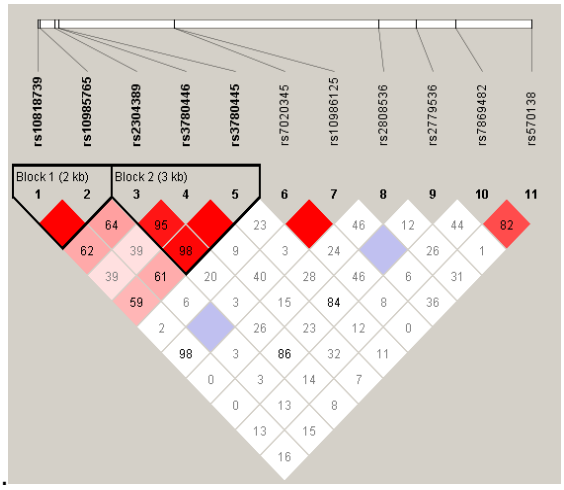
Table 9: Four SNPs with its genes and proteins they encode for are shown in this table. Genes coding for proteins involved in receptor activity, its ligands are also illustrated. The fifth column represents the relationship between the SNP and its effect on the self-reported pain perception, either is the SNP associated with a reduction –rs1888861 TrkA- or an increase –rs5224 BDKRB2 and rs7709656 GLRA1- in pain report.

3.5 Replication of data

Replication of our results using a different cohort was however not possible for each specific SNPs. Haploview enabled us to display which SNPs are in strong linkage disequilibrium –meaning the likelihood of being inherited together is high- with our reference SNPs, Figure 12. Using these SNPs with a strong LD, replication nonetheless was still not possible. P-values for SNPs in strong LD did not appear to be significant in the second cohort. To double check, we have marked SNPs, which were close to a significance level in the second cohort and looked at the strength of LD between those and our SNPs. This still did not achieve any possible results for replication.

12a. 

12b. 

12c. 

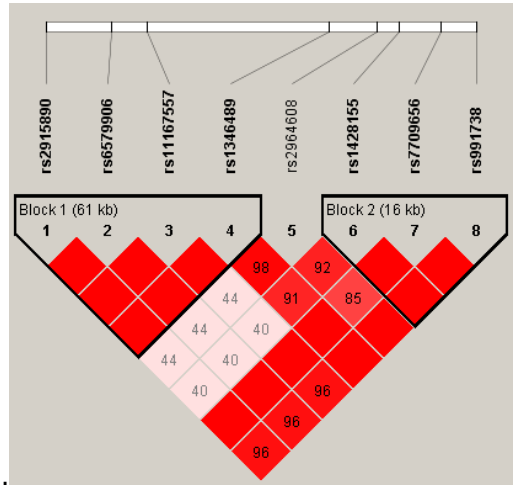
12d. 

Figure 12: a. LD-plot for rs188861 in TRKA. b. LD-plot for rs5224 in BDKRB2. c. LD-plot for rs3780446 in GABBR2. d. LD-plot for rs7709656 in GLRA1

3.6 Lambda

A sum-up of outcomes for Lambda is shown below in Table 10 indicating the absence of any systematic bias in our analyses.

Covariates	***Lambda λ***
None	1.01
Age	0.993146
History of previous surgery (preop)	1.01583
BMI	1.00975
Age, Preop	1.01592
Age, Preop, BMI	1.01535

Table 10: Λ was calculated using PLINK. Results show that our genomic inflation factor for each regression test is very close to 1. This suggests, that there is sufficient power and only very little confounds in our statistic, replicating what has been already observed by a close adherence on the Q/Q-plots.

3.7 Possible association between found SNPs and treatment response with ACS

The subchapters above describe an association between pain report in KOA patients and four SNPs. Along to this analysis, the question came up if these SNPs associated with pain report can also be associated with treatment response with ACS. To enable a comparison a linear regression analysis for treatment response only including patients treated with ACS was carried out.

SNP	***Gene***	***Chr***	***Location on Chr (bp)***	***p-value (VAS)***	***p-value (WOMAC)***
rs5224	BDKRB2	14	95777210	0.2731	0.3157
rs3780446	GABBR2	9	100111383	0.5872	0.6422
rs7709656	GLRA1	5	151270918	0.9744	0.6945
rs1888861	TRKA	1	155064585	0.9439	0.9311

Table 11: This table displays results from a linear regression analysis only for the four SNPs, which were most significant in regression analysis for pain report. Again, regression analysis has been carried out twice, using VAS and WOMAC data. The two columns on the right stand for the significance niveau.

p-values of this analysis are far off being significant, hence there was no association in our regression analysis between the four mentioned SNPs and treatment response to ACS. Imminent of this, these SNPs cannot be used as predictive markers for treatment response in KOA.

4. Treatment response

As mentioned at the start, it is hypothesized that certain SNPs exist that can be associated with a treatment response to ACS, HA and saline as placebo. In the first part of our analysis we have found out that SNPs, which are associated with pain reports do not correlate with treatment response to ACS (Table 11). This chapter of the thesis will lay its focus upon the evaluation of regression analyses and comparison of SNPs for treatment response to the three subjects ACS, HA and saline. Treatment response was calculated by the difference of baseline pain rating in relation to pain rating at week 26. It should be kept in mind that baseline pain rating could act as a possible confounding variable in this set of regression analyses.

4.1 Treatment response to ACS

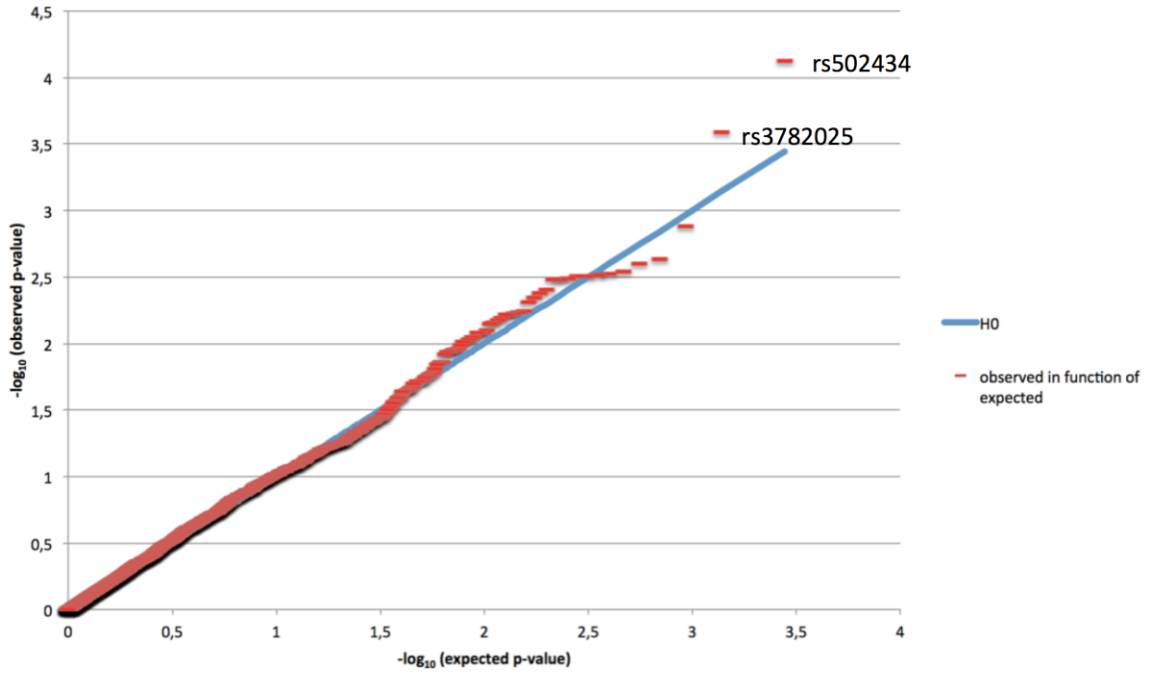
First, we carried out a regression analysis for treatment response with ACS. The results of the five most significant SNPs are displayed in table 12. Nor SNPs or genes overlap with relevant SNPs in pain report -from the first part of this dissertation-.

Results for the five most significant SNPs of regression analysis using WOMAC and VAS data for treatment response to ACS.

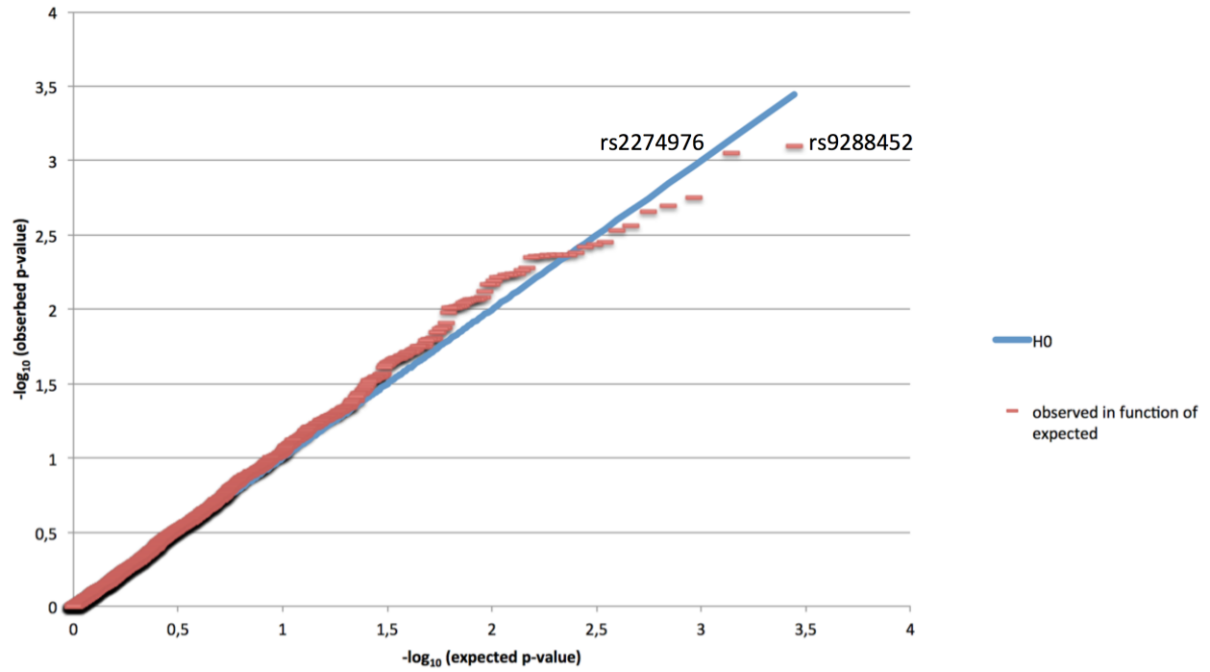
SNP	***Gene***	***Chr***	***p-value***	***Beta***
WOMAC				
rs502434	GRIA3	23	7.55E-05	1.399
rs3782025	HTR3B	11	0.0002558	-1.133
rs1518111	IL10	1	0.001306	-1.26
rs624945	STAU1	20	0.002322	1.595
rs9610496	MAPK1	22	0.002519	-1.208
VAS				
rs9288452	ERBB4	2	0.0007988	1.456
rs2274976	MTHFR	1	0.0008833	3.881
rs9610496	MAPK1	22	0.00178	-1.331
rs529520	OPRD1	1	0.002007	-1.144
rs2391333	EFNB2	13	0.002217	-1.13

Table 12: SNPs in table above are sorted in descending order by p-value when adjusting for WOMAC. As it can be observed rs502434 located in GRIA3 –coding for glutamate ionotropic

receptor AMPA type subunit 3- is very close to passing the Bonferroni correction, implying a possible influence in treatment response to ACS.



13a. WOMAC



13b. VAS

Figure 13: Q/Q-plot graphically displaying two SNPs above the Null-Hypothesis H0. H0 is being calculated through expected p-value in function of the expected p-value In the top right corner is rs502434.

Subsequently further regression tests were carried out to see whether SNPs from table 12 can also be associated with treatment response under HA and saline. Comparison of 5 most significant SNPs in treatment response under ACS with treatment response under HA and saline show no correlation as it is graphed in table 13.

SNP	***Gene***	***Chr***	***p-value for ACS***	***p-value for HA***	***p-value for Saline***
rs502434	GRIA3	23	7.55E-05	0.3005	0.7309
rs3782025	HTR3B	11	0.0002558	0.4685	0.8893
rs1518111	IL10	1	0.001306	0.5566	0.9923
rs624945	STAU1	20	0.002322	0.8947	0.4397
rs9610496	MAPK1	22	0.002519	0.8022	0.1359

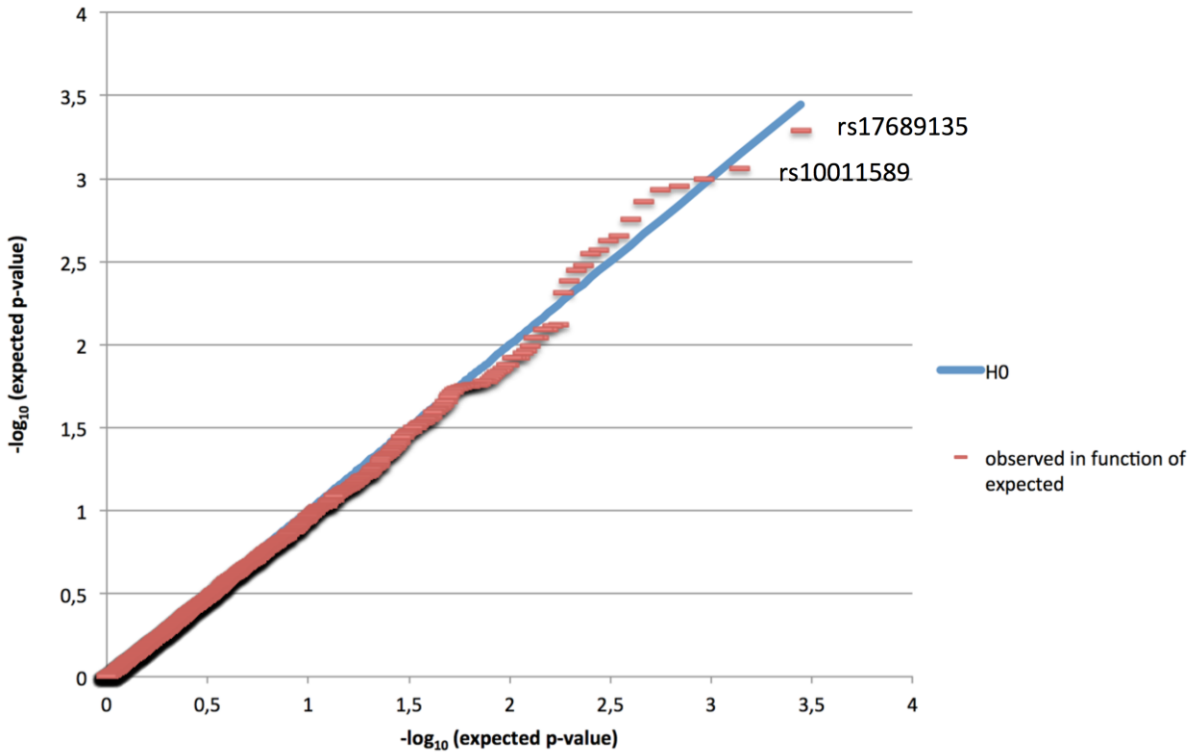
Table 13: SNPs arranged in descending order of p-values for treatment response under ACS. The five most significant SNPs found in treatment response under ACS show no association, which could be explained using the results of the regression analysis with treatment responses under HA or saline.

4.2 Treatment response to HA

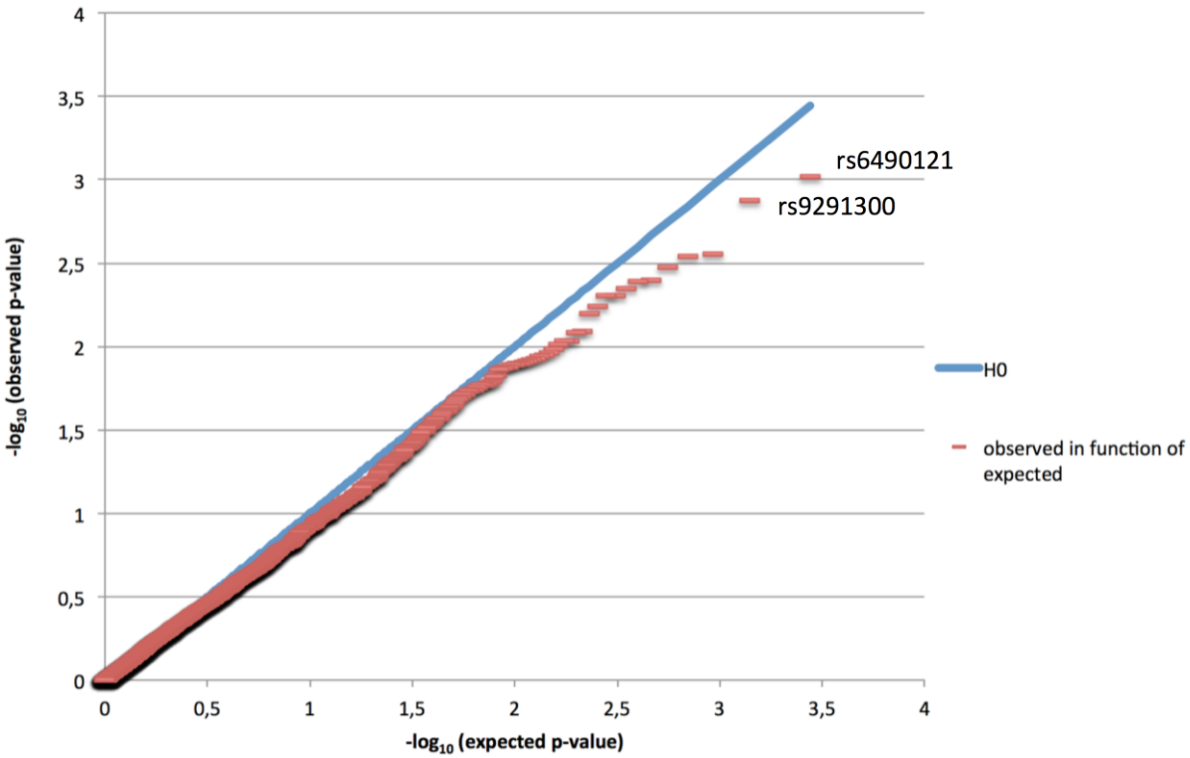
Results for the five most significant SNPs of regression analysis using WOMAC and VAS data for treatment response to HA

SNP	***Gene***	***Chr***	***p-value***	***Beta***
WOMAC				
rs17689135	VPS4B	18	0.0005152	-1.38
rs10011589	GRIA2	4	0.0008672	1.255
rs10517665	GRIA2	4	0.001005	1.388
rs1549758	NOS3	7	0.001121	1.067
rs806368	CNR1	6	0.00117	-1.337
VAS				
rs6490121	rs6490121	12	0.000962	1.176
rs9291300	rs9291300	4	0.001327	-1.578
rs17058952	rs17058952	8	0.002796	-1.107
rs2293054	rs2293054	12	0.0029	1.17
rs3792208	rs3792208	4	0.003371	1.17

Table 14: rs17689135 and rs10011589 for WOMAC and rs6490121 for VAS show a slight significance in treatment response with HA in KOA patients, acknowledging bias opinions on the effect of HA. Jevsevar et al have concluded, based on a broad literature research, that there is no clinically important difference of injectable HA over PL in KOA patients (Jevsevar, Donnelly, Brown, & Cummins, 2015).



14a. WOMAC



14b. VAS

Figure 14a, 14b: Illustrating WOMAC and VAS results from Table 14 on a Quantile-Quantile-Plot gives graphical evidence of absence passing the significance threshold in treatment response under HA, since SNPs adhere close to H0.

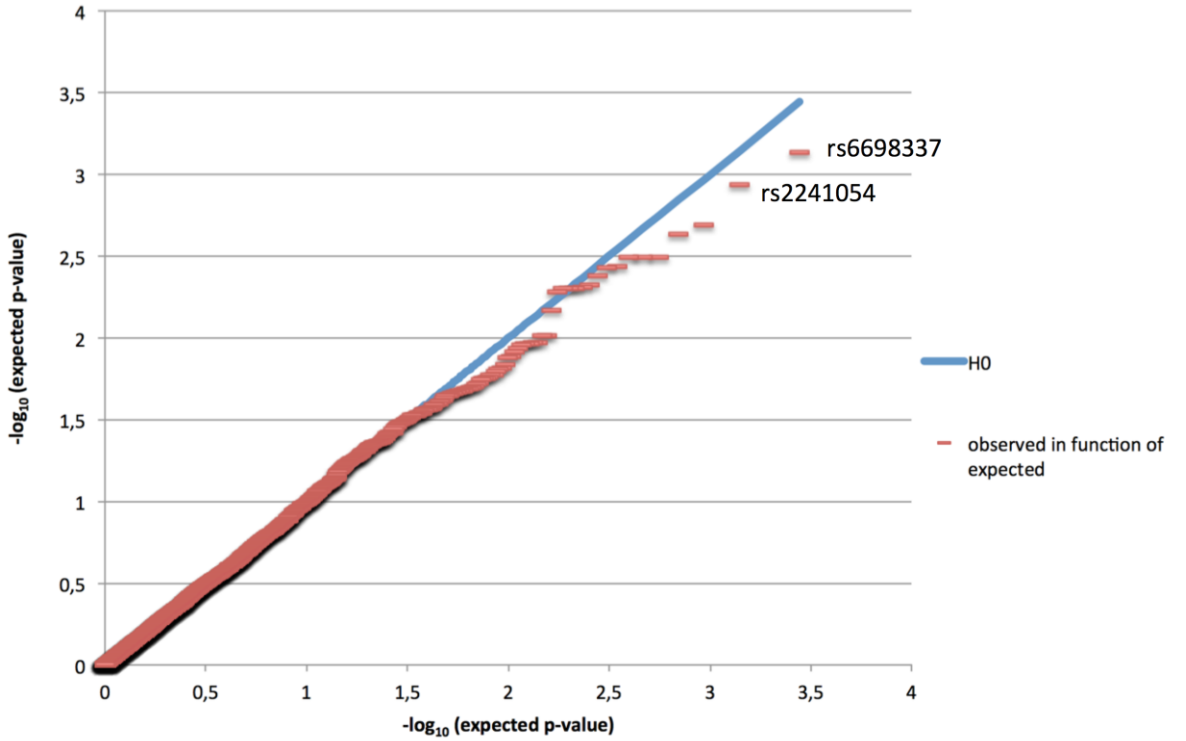
4.3 Treatment response to PL

Results for the five most significant SNPs of regression analysis using WOMAC and VAS data for treatment response to PL.

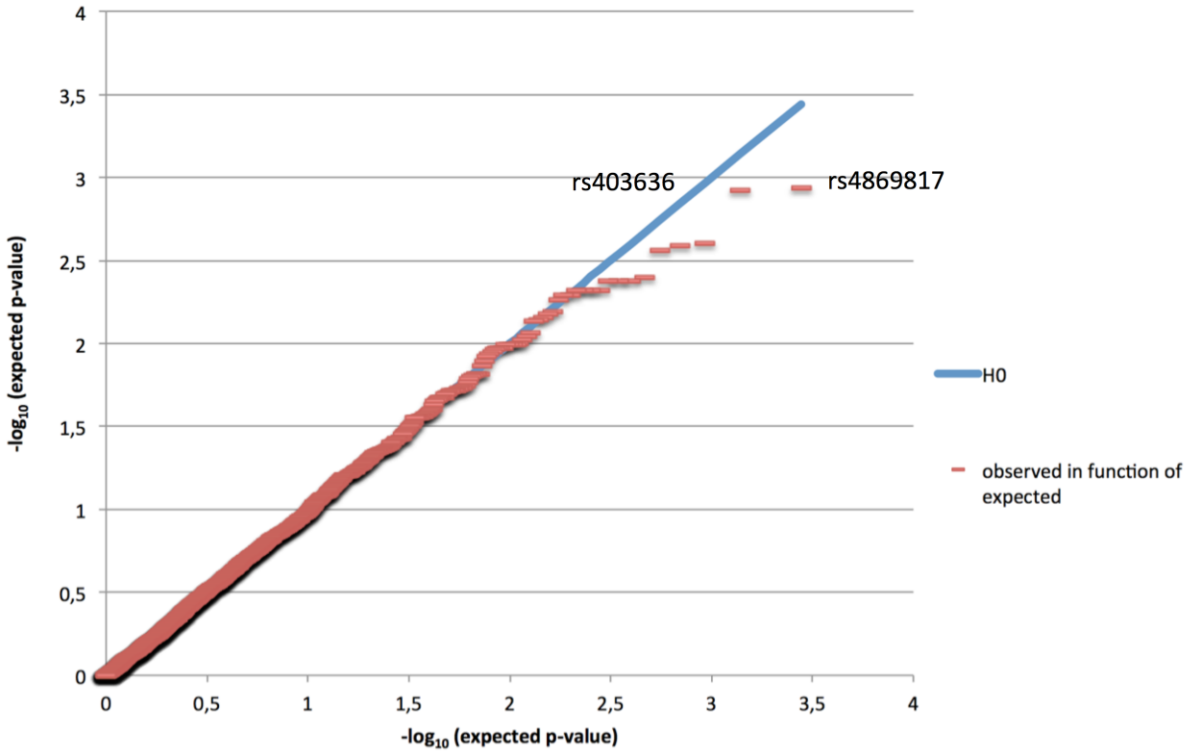
SNP	***Gene***	***Chr***	***p-value***	***Beta***
WOMAC				
rs6698337	INADL	1	0.0007347	1.274
rs2241054	EGFR	7	0.001153	1.124
rs5917471	CYBB	23	0.00203	1.303
rs11543848	EGFR	7	0.002313	1.281
rs2239448	MAOA	23	0.003196	-1.183
VAS				
rs4869817	OPRM1	6	0.001161	1.355
rs403636	SLC6A3	5	0.001203	1.951
rs12936511	CRHR1	17	0.002472	-2.608
rs3750717	CPN1	10	0.002582	-3.592
rs3729910	MAPK1	22	0.002735	-2.902

Table 15: An association with p = 0.00074 between rs6698337 and treatment response to PL was observed when WOMAC data was applied. No further SNPs were associated with treatment response under application of WOMAC data.

Regression analysis showed that no SNPs caused a significant difference in clinical appearance under PL when VAS was applied. Strongest association between a SNP and treatment response to PL lay at a p = 0.001161 for rs4869817 of OPRM1, which is far off from our Bonferroni-corrected p value of $p < 1.892e^{-5}$.



15a. WOMAC



15b. VAS

Figure 15: SNPs for treatment response under saline acting as our placebo do not pass the significance niveau for a clinical relevance. All SNPs are located very closely to H0, implicating no correlation between SNPs and PL treatment response.

4.4 Outline of main points

Table 16, 17, 18 and 19 outline the main points found in our study regarding Hypothesis 1 and 2. For all regression tests carried out and mentioned in this thesis, distribution on Q/Q-plots and Lambda as a control for population stratification implied no systematic deviation.

Association between SNPs and pain report			
Phenotype Data	***WOMAC***		***VAS***
SNP	**rs5224**	**rs7709656**	**rs1888861**
Gene	BDKRB2	GLRA1	TRKA
Chr	14	5	1
Loaction of Chr (bp)	95777210	151270918	55064585
Pain Panel SNP	C/T	A/G	A/G
Minor allele	T	A	G
MAF	0.128	0.173	0.190
p-value	0.0001194 – 0.0002919	0.0003759 – 0.0006763	0.0000986 – 0.0001526
Beta	4.749 – 4.999	4.613 – 4.799	-5.010 – -5.206
Effect on pain	↑	↑	↓

Table 16: Please see next page for description of table 16.

Table 16: Recap of association results for the top-ranked SNPs in pain report in 272 midstage KOA patients. The significance threshold after a spectral decomposition analysis – to find the effective number of independent SNPs- was set to $p < 1.892e^{-5}$. The listed SNPs come close to the significance threshold, but do not pass it. Table 16 displays range of significance niveau p for regression analysis in absence as well as in presence of covariates; p values do not diverge strongly within these analyses. Minor alleles of rs5224 C>T -T being the minor allele- (p 0.00029) and rs7709656 A/G -A being the minor allele- (p 0.0005) are associated with an increased pain report in KOA, whereas minor alleles of rs1888861 A>G – minor allele being G- (p 0.00015) correlate with a lowered pain perception.
Minor allele G of rs1888861 ($p = 9.86e^{-05}$) nearly passes the significance threshold in a regression test without any covariates applied.
The row with p-values shows the range of results for all five regression tests carried out - applying all covariates, each covariate separately and no covariates applied-.
MAF displays the frequency of presence of the minor allele of a SNP.

Association between SNPs and altered treatment response to ACS				
Phenotype Data	***WOMAC***		***VAS***	
SNP	**rs502434**	**rs3782025**	**rs9288452**	**rs2274976**
Gene	GRIA3	HRT3B	ERBB4	MTHFR
Chr	23	11	2	1
Loaction of Chr (bp)	122364958	151270918	212895796	11773514
Pain Panel SNP	A/G	C/T	C/T	C/T
Minor allele	A	C	T	T
MAF	0.402	0.465	-	0.062
p-value	$7.55e^{-5}$	0.0002558	0.0007988	0.0008833
Beta	1.40	-1.13	1.456	3.881
Effect on treatment response	↑	↓	↑	↑

Table 17: Outline of results collected for Hypothesis 2 is displayed. Under application of WOMAC phenotypic data, the minor allele A of rs502434 (glutamate ionotropic receptor AMPA type subunit 3) –with a frequency of 0.402- strongly correlates with response to ACS treatment with a significance of $p = 7.55e^{-5}$, nearly passing the Bonferroni threshold. Further, minor allele C of rs3782025T>C (5-HT receptor 3B) –with a frequency of 0.465-, is associated to treatment response with a significance niveau of $p = 2.56e^{-4}$. When VAS phenotype was applied, two different SNPs, rs9288452 and rs2274976 with a MAF of 0.009 - minor allele T- appeared to correlate with an altered treatment response to ACS.
MAF for rs9288452 C>T could not be calculated.

Association between SNPs and altered treatment response to HA			
Phenotype Data	***WOMAC***		***VAS***
SNP	**rs17689135**	**rs10011589**	**rs6490121**
Gene	VPS4B	GRIA2	NOS1
Chr	18	4	12
Loaction of Chr (bp)	59236106	158377702	116192578
Pain Panel SNP	G/T	A/T	A/G
Minor allele	T	A	G
MAF	0.239	0.158	0.358
p-value	0.0005152	0.0008672	0.000962
Beta	-1.38	1.26	1.18
Effect on treatment response	↓	↑	↑

Table 18: Correlations between the presence of minor alleles of 3 SNPs and varied treatment response to HA were found. P-values do not reach significance level and are less significant than results for ACS treatment response.

Association between SNPs and altered treatment response to PL	
Phenotype Data	***VAS***
SNP	**rs6698337**
Gene	INADL
Chr	1
Loaction of Chr (bp)	62110395
Pain Panel SNP	A/G
Minor allele	A
MAF	0.456
p-value	0.0007347
Beta	1.27
Effect on treatment response	↑

Table 19: Only on SNP was found when carrying out regression tests to evaluate correlations between SNPs and alteration in treatment response to saline (PL). when using VAS phenotypic data, the presence of the minor allele A rs6698337G>A INADL was associated with a better treatment response to PL.

5. Discussion

This chapter will first outline an attempt at translating the statistical results into clinical relevance and application. Besides, the chapter will focus on possible consequences on molecular level and alteration within pathways caused by SNPs and will go into further detail on gene analysis. Furthermore, an initial attempt of genetic involvement in pain report is made.

Interpretation of statistical results with regard to clinical relevance for Hypothesis 1 Pain perception in KOA:

- BMI, age and history of previous surgery in KOA patients cannot be used to better predict a patient's pain perception in KOA
- Presence of the minor allele T of rs5224 C>T BDKRB2 as well as the presence of minor allele A of rs7709656 G>A GLRA1 are risk factors for an increased pain perception in KOA
- Presence of the minor allele G of rs1888861 A>G TrkA protects against pain in KOA

Interpretation of statistical results with regard to clinical relevance for Hypothesis 2 Treatment response to ACS:

- Patients with a present minor allele A of rs502434 G>A GRIA3 show an enhanced treatment response under intra-articular injected ACS against KOA, whereas patients with the minor allele C of rs3782025 T>C HRT3B show a reduced effect of injected ACS against their KOA
- Correlations between these SNPs and treatment response were strong ($7.55e^{-05} < p > 2.56e^{-04}$), nearly passing significance threshold of $p < 1.892e^{-5}$

Treatment response to HA:

- Patients with the minor allele A of rs17689135 G>T VPS4B show a reduced response to intra-articular injected HA, whereas patients with the minor allele A of rs10011589 T/A GRIA2 and minor allele G of rs6490121 A>G NOS1 had a better response to HA treatment
- Kept in mind that results of the association studies for HA treatment response did not pass the significance niveau ($0.0005 < p > 0.000962$) and did not come as close as results for ACS treatment response did

Treatment response to PL:

- The presence of the minor allele A of rs6698337 G>A INADL may be associated with an increased treatment response under saline.
- Again, results did not pass the significance threshold (p = 0.0007) and did not come as close to the threshold as results for ACS did

5.1 Possible consequences of a SNP

A SNP can occur in three different types of location within gene loci. The following will describe the aftereffect a SNP´s positioning might have within certain loci:

1. Promoter region: the promoter region is positioned upstream from a gene toward 5' (Sharan, 2007). It regulates transcription rate by acting as a secure DNA binding site for the RNA polymerase. The promoter region can be sub grouped into a core promoter, a proximal and a distal promoter (Juven-Gershon, Hsu, Theisen, & Kadonaga, 2008): The Core promoter is made up of binding sites for RNA polymerases I-III and transcription factor binding sites, such as TATA-boxes. The proximal and distal promoters bind specific transcription factors. Once RNA polymerase and other necessary factors bind the promoter, transcription is initiated (Smale & Kadonaga, 2003). Further, transcription factors can bind the promoter and can either function as activators or repressors of transcription (J. D. Watson, 2013). SNPs within this region can cause an augmented or reduced transcription rate for a protein, which would lead to a quantitative change of the encoded protein.
2. Coding region: the coding region is made up of exons, which carry genetic information for the mature RNA product, and introns. Non-coding Introns will be excluded during RNA transcription. This process is called RNA splicing. Polymorphisms within the coding region can therefore either be located within an intron or an exon. SNPs within an exon can cause altered functioning of a protein in different ways. A non-synonymous change –meaning the triplet codes for a different amino acid- will generate a variation in protein structure; whereas a synonymous change –meaning the encoded amino acid does not vary- may also impact downstream. 64 codons exist (61 coding for an amino acid and 3 stop codons, terminating translation) and only 20 different amino acids exist. Codons can be grouped into major and minor codons depending

on the present tRNA abundance. Through codon usage[13], synonymous variants may have an impact upon mRNA secondary structure, regulating gene expression, speed of translation and protein folding downstream (Cortazzo et al., 2002; Kimchi-Sarfaty et al., 2007; Novoa & Ribas de Pouplana, 2012; Spencer & Barral, 2012).

Intronic as well as exonic variants may regulate a genes expression by alternative splicing. SNPs within splicing enhancer regions can down and up regulate certain splicing variations (Guo et al., 2014).

3. 3' UTR (three prime untranslated region): downstream of the gene lays the three-prime region which does not code for the mature RNA product, however still plays an important role in gene expression. Stabilization –for example via polyadenylation-, export and translation efficiency is regulated by this region, which can be influenced by SNPs.

   MREs represent microRNA response elements, to which posttranscriptional gene-regulatory miRNA can bind. Numerous mechanisms exist –including binding mRNA at MREs, destabilizing mRNA, inhibiting polyadenylation or acting as initiation blocks for ribosome assembly-, where miRNA play a key role leading to translational repression, meaning the observation of a greater decrement in protein product compared to the decrement of levels of mRNA (Baek et al., 2008; Gu & Kay, 2010).

   AU-rich elements in the 3' UTR region display binding sites for proteins, which again can affect mRNA degradation and translation rate (Barrett, Fletcher, & Wilton, 2012).

   Further sequence involved in affecting mRNA's stabilization, export and translational efficiency is the Poly(A)tail, to which gene-regulatory proteins can bind. Polyadenylation is associated with a translation-activating characteristic, whereas the absence is associated with a decay of mRNA (Wilusz, Wormington, & Peltz, 2001).

[13] Codon usage describes the usage of synonymous codons at different frequencies within the genome varying from species to species (Plotkin & Kudla, 2011).

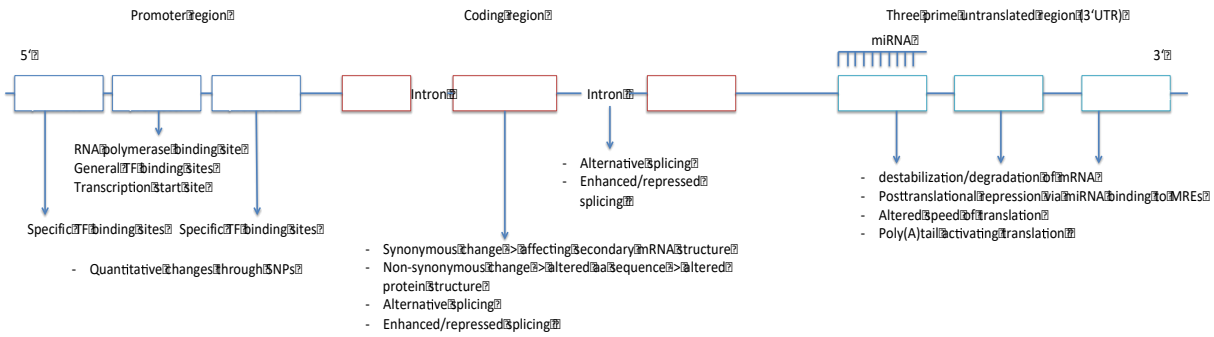


Figure 16: This illustration depictures possibilities of SNPs positioning with its result on transformation and protein structure as described above.
MREs: microRNA response elements
AREs: AU-rich elements
TF: transcription factors
miRNA: microRNA

Overall, SNPs in all three regions of a locus can have a quantitative as well as a qualitative alteration of mRNA and downstream protein as a consequence.

With the help of NCBI[14], our SNPs could be associated to regions within their encoded gene. It is an initial attempt to seek an idea of how our SNPs cause a change in protein functioning and affect pain report in KOA.

SNP	***Gene***	***Position on Chr (bp)***	***Region***	***Consquence***
rs1888861	TrkA	155,064,585	coding region	intron variant
rs5224	BDKRB2	95,777,210	-	synonymous codon (Thr264Thr)
rs7709656	GLRA1	151,270,918	coding region	intron variant
rs3780446	GABBR2	100,111,383	coding region	intron variant

Table 20: SNPs within the three genes TrkA, GLRA1, GABBR2 lead to an intronic variant within the DNA sequence. Rs5224 prompts to a synonymous codon, meaning the amino acid sequence does not differ. Synonymous codon rs5224: ACG -> ACA both coding for threonine with a frequency of use of 6.11 and 15.1 in 1000bp (data from Codon Usage Database, which uses NCBI-GenBank)

[14] National Center for Biotechnology Information

5.2 Analysis of SNPs estimated to be involved in treatment response under ACS

SNP	***Gene***	***Protein***	***Ligand/ Function of protein***	***SNP's effect***	***Effect on treatment response (beta)***
WOMAC					
rs502434	GRIA3	ionotropic glutamate receptor AMPA type subunit 3	Glutamate	synonymous codon	↑
rs3782025	HTR3B	5-hydroxytryptamine receptor type B	Serotonin	intron variant	↓
VAS					
rs9288452	ERBB4	Erb-b2 receptor tyrosine kinase 4	Epidermal growth factors (EGF)	Intron variant	↑
Rs2274976	MTHFR	methylenetetrahydrofolate reductase	Enzyme[15]	Intron variant (missense)	↑

Table 21: The presence of two of the index SNPs rs502434 and rs624945 are allied with an increment in treatment response to ACS, meaning a better score in WOMAC and a lower score VAS. Slade et al have shown that raised levels of IL 8 and other cytokines correlate with the sensitivity to pain in patients with TMD –temporomandibular disorder- (Slade et al., 2011) implicating that pro-inflammatory substances have an impact upon chronic pain disorders.

Using web based research via NCBI, pathwayLinker and results from the regression analysis via PLINK further information has been collected which is being displaying in Table 19. This information includes the protein the genes encode for,

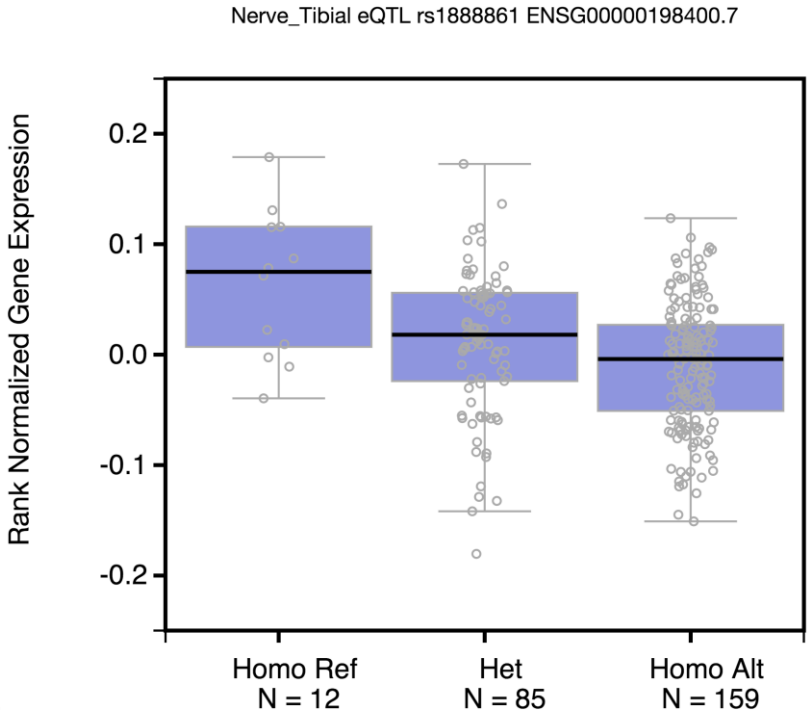
[15] Converting 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is a co-substrate in the remethylation from homocysteine to methionine

their ligands or functions of the protein, the SNPs effect within the genome and its effect on treatment response to ACS.

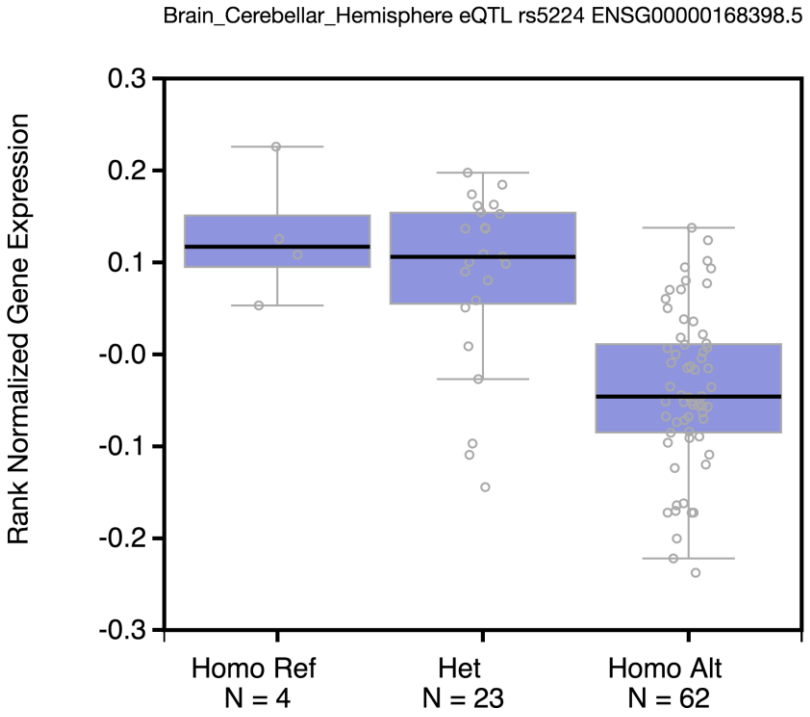
rs502434 is located on the X-chromosome within GRIA3, encoding for the subunit 3 of the ionotropic glutamate receptor of AMPA type. AMPA acts as an excitatory receptor in neurophysiological pathways in the mammalian brain. Literature suggests that SNPs within GRIA1 and GRIA3 play a critical role in cortical depression and migraine with and without aura. (Formicola et al., 2010). Further literature check on GRIA gave insight on a novel idea implicating AMPA receptors as possible targets in therapy for pain-induced depression. Goffer et al suggest that enhancement of AMPA in the nucleus accumbens can reduce depression-like behavior in chronic pain conditions. (Goffer et al., 2013). Approaching with the idea of a multifactorial origin of pain including a psychological component, it should be kept in mind that depression-like behavior can enhance the sensation of pain and hence the subjective sensation of response to a certain treatment. Results in our study have shown that the presence of the minor allele of SNP rs502434 correlates with a better response to ACS.

5.3 SNP effects on gene expression

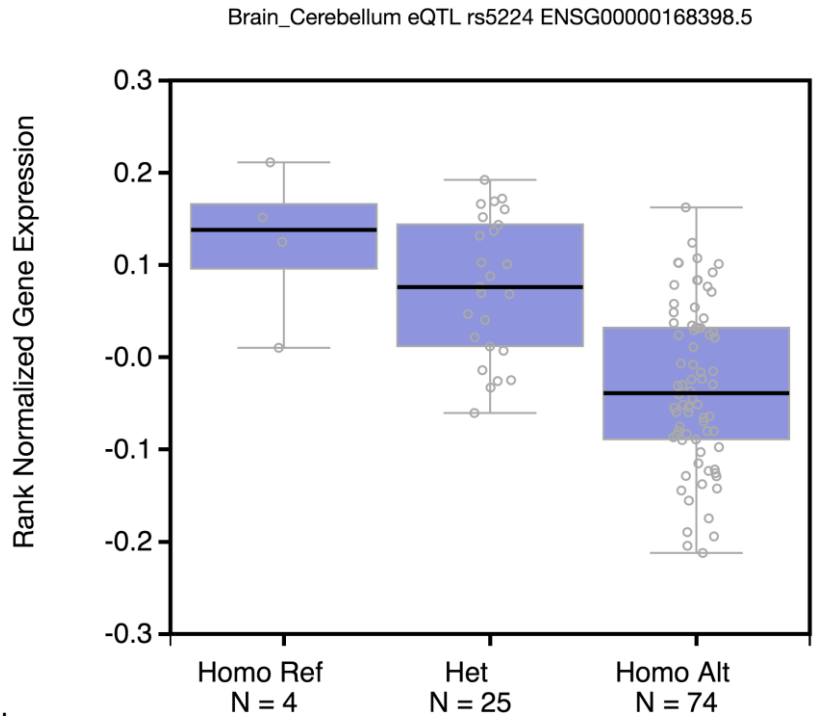
The web based tool GTex enabled us to i) look up if a SNP has an effect on gene expression and ii) in what tissue the association was found in, described as expression quantitative trait locus (eQTL). Sadly, we only found data about gene expression in interesting tissues for two of our SNPs, rs1888861 and rs5224. GTex did not include data for other SNPs involved in alteration of pain perception and treatment response.


Nerve_Tibial eQTL rs1888861 ENSG00000198400.7
Rank Normalized Gene Expression
0.2
0.1
0.0
-0.1
-0.2
Homo Ref
N = 12
Het
N = 85
Homo Alt
N = 159

17a.


Brain_Cerebellar_Hemisphere eQTL rs5224 ENSG00000168398.5
Rank Normalized Gene Expression
0.3
0.2
0.1
-0.0
-0.1
-0.2
-0.3
Homo Ref
N = 4
Het
N = 23
Homo Alt
N = 62

17b.



17c.

Figure 17: Gene expression levels by genotype. Box plots designed by GTex with a sample size with genotyping n=7333 of a donor set n=449 (Consortium, 2013; http://www.gtexportal.org/home/).

a) Rs1888861: Selected tissue tibial nerve. In presence of a homozygous genotype of the minor allele G/G n=12 an enhanced gene expression is measured. The heterozygous genotype only results in a slight increase in TrkA expression. Rs1888861 appears to follow a recessive genetic trait, because only the presence of both minor alleles causes an augmentation in gene expression.
b) Rs5224: selected tissue cerebellum. The homozygous genotype of the minor allele T/T n=4, as well as the heterozygous genotype n=23, leads to a nearly equally great increment in BDKRB2 expression, indicating a dominant genetic trait, since the occurrence of one minor allele already has an impact upon gene expression, when cerebellar tissue is analyzed.
c) Rs5224: selected tissue cerebellar hemisphere. The Homozygous genotype of the minor allele T/T n=4 generates a greater augmentation of gene expression than the heterozygous genotype n=25. The homozygous genotype of the major allele C/C n=74 is associated with a decrement in gene expression of BDKRB2.

SNP	***homozygote genotype minor allele***	***minor allele homozygote genotype frequency***	***heterozygote genotype frequency***	***alternative allele homozygote genotype frequency***
rs5224 (C/T)	T/T	0.018	0.221	0.761
rs7709656 (A/G)	A/A	0.035	0.274	0.690
rs1888861 (A/G)	G/G	0.053	0.274	0.673

Table 22: Data taken from the international HapMap Project of 90 DNA samples from a Utah US population with Northern and Western European ancestry (CEU) (International HapMap, 2003) functioning as a representation for genotype frequencies in our Caucasian cohort. In table 22 the 3 possible genotype frequencies for an allele of the genes found to be involved in pain perception in KOA are displayed.

5.4 Pathway analysis for genes found to be involved in pain report

Accompanying the search of the altered protein structure, a pathway analysis may give a greater insight on how genes, on which the four SNPs are located, interact and in which signaling pathways they are known to be involved in. A Pathway Software, PathwayLinker, enabled us to carry out separate searches for each of our four genes, GABBR2, TRKA, BDKRB2, as well as displaying certain interactions in different pathways.

5.4.1 Pathway analysis for TrkA

Tropomyosin receptor kinase A was found to be involved in a handful of pathways, including pathways in -cancer thyroid cancer (Frattini et al., 2004)-, apoptosis as well as MAPK signaling pathway (Figure 18), neurotrophin signaling pathway (Figure 19) and inflammatory mediator regulation of TRP channels, which will be explained in more detail further along this dissertation.

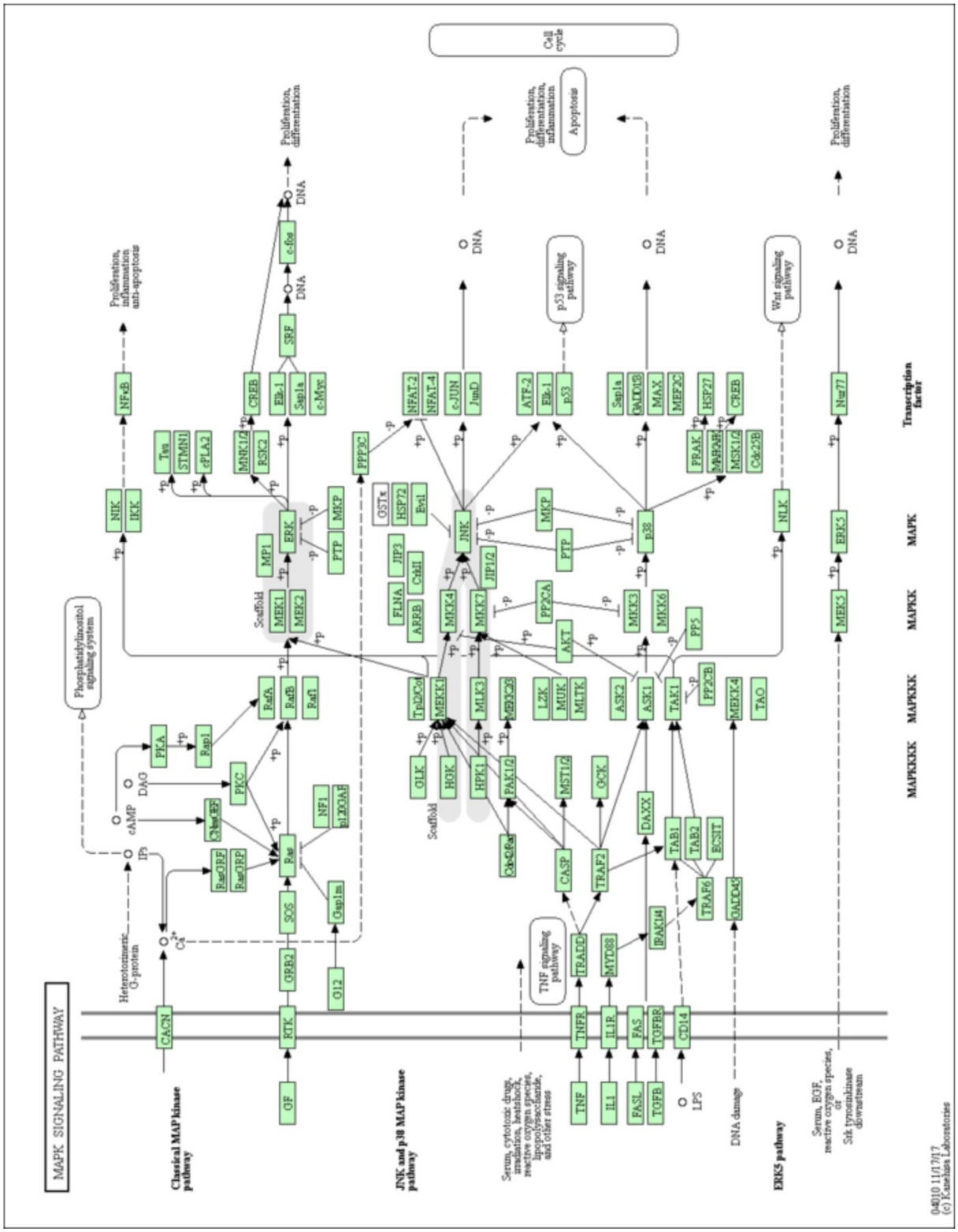


Figure 18[16]: RTK stands for receptor tyrosine kinase, GF for Growth factors, to whom NGF, BNDF and NT3, 4 (being subgroups of NGF) belong. TrkA (displayed as RTK) is activated when NGF (nerve growth factor) (displayed as GF), BDNF (brain-derived neurotrophic factor) and NT3, 4 (Neurotrophin 3, 4) bind the receptor, which all belong to the family of neurotrophins. A cascade of phosphorylation via Ras, Raf, MEKK and MEK leads to an

[16] http://www.genome.jp/kegg-bin/show_pathway?scale=0.67&query=&map=hsa04010&scale=0.82&auto_image=&show_description=hide&multi_query=

activation of ERK (Extracellular signal-regulated kinase). cPLA2 (cytosolic phospholipase A2) furthermore participates in TRP channel activation, which was found out to be another intersection of our most significant genes (Vriens et al., 2004).

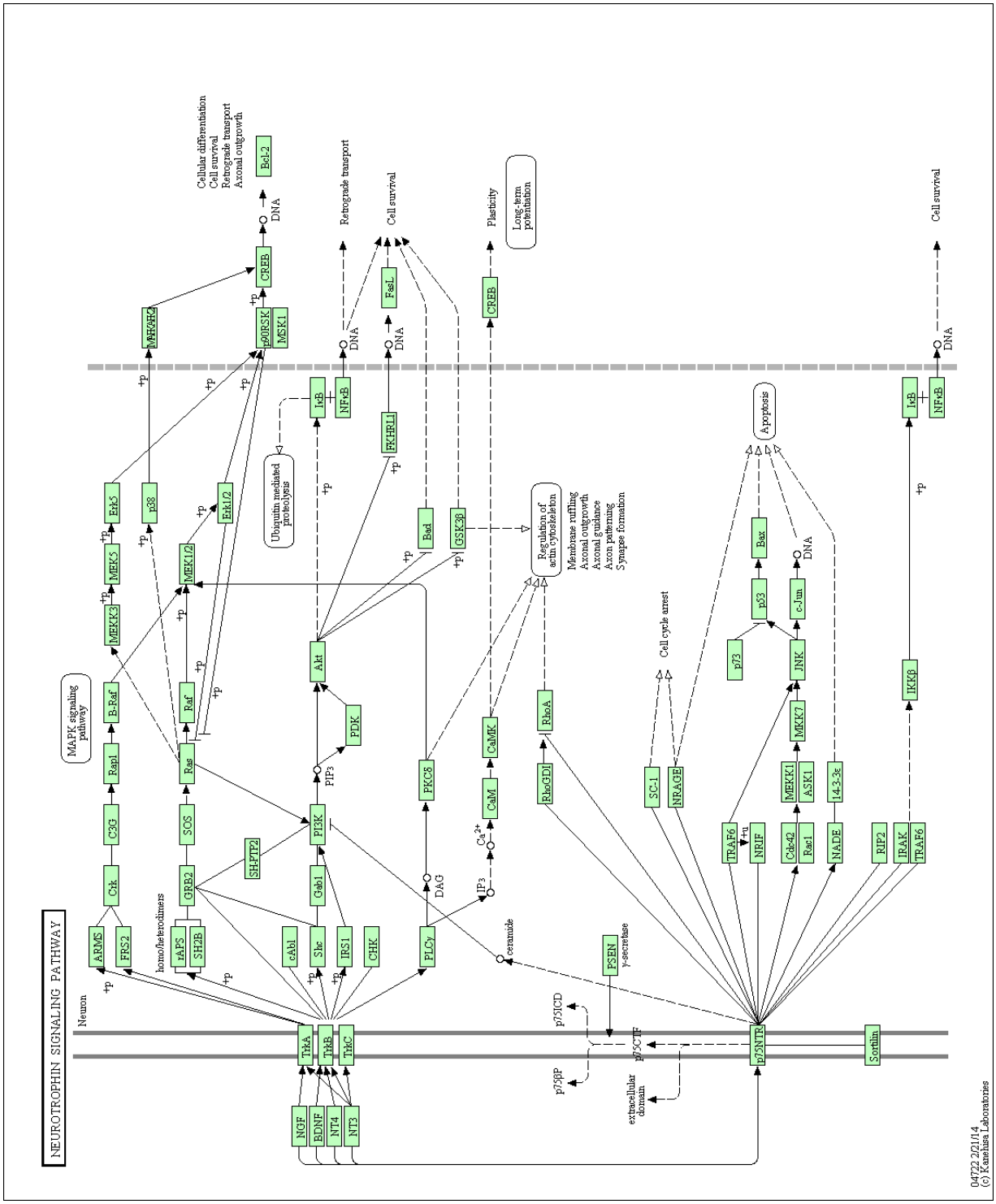


Figure 19[17]: Neurotrophin-mediated dimerization and phosphorylation of a certain loop of the Trk receptors activate these receptors (Huang & Reichardt, 2003). TrkA has been found to be involved in the neurotrophin signaling pathway, via the MAPK pathway it results in a regulation of neurite cellular differentiation, functioning and outgrowth (Reichardt, 2006).

[17] http://www.genome.jp/kegg-bin/show_pathway?hsa04722

5.4.2 Pathway analysis for BDKRB2

BDKRB2 is located on the long arm of chromosome 14 –exact location is 14q32.1-32.2- and encodes a receptor for Bradykinin, a 9aa long peptide. Bradykinin evokes responses including vasodilatation, edema, smooth muscle spasm and pain fibre stimulation (http://www.ncbi.nlm.nih.gov/gene/624). Presence of the Minor allele of rs5224 C/T -T being the minor allele- has been associated with a greater pain report in patients with KOA in this analysis.

BDKRB2 plays a role in the activation of the cGMP-PKG signaling pathway. This pathway is also connected to the MAPK signaling pathway (Figure 20) as well as the phosphatidylinositol signaling system, which is involved in membrane trafficking, hence it can have an influence upon levels within the cell, the release of proteins and internalization from extracellular.

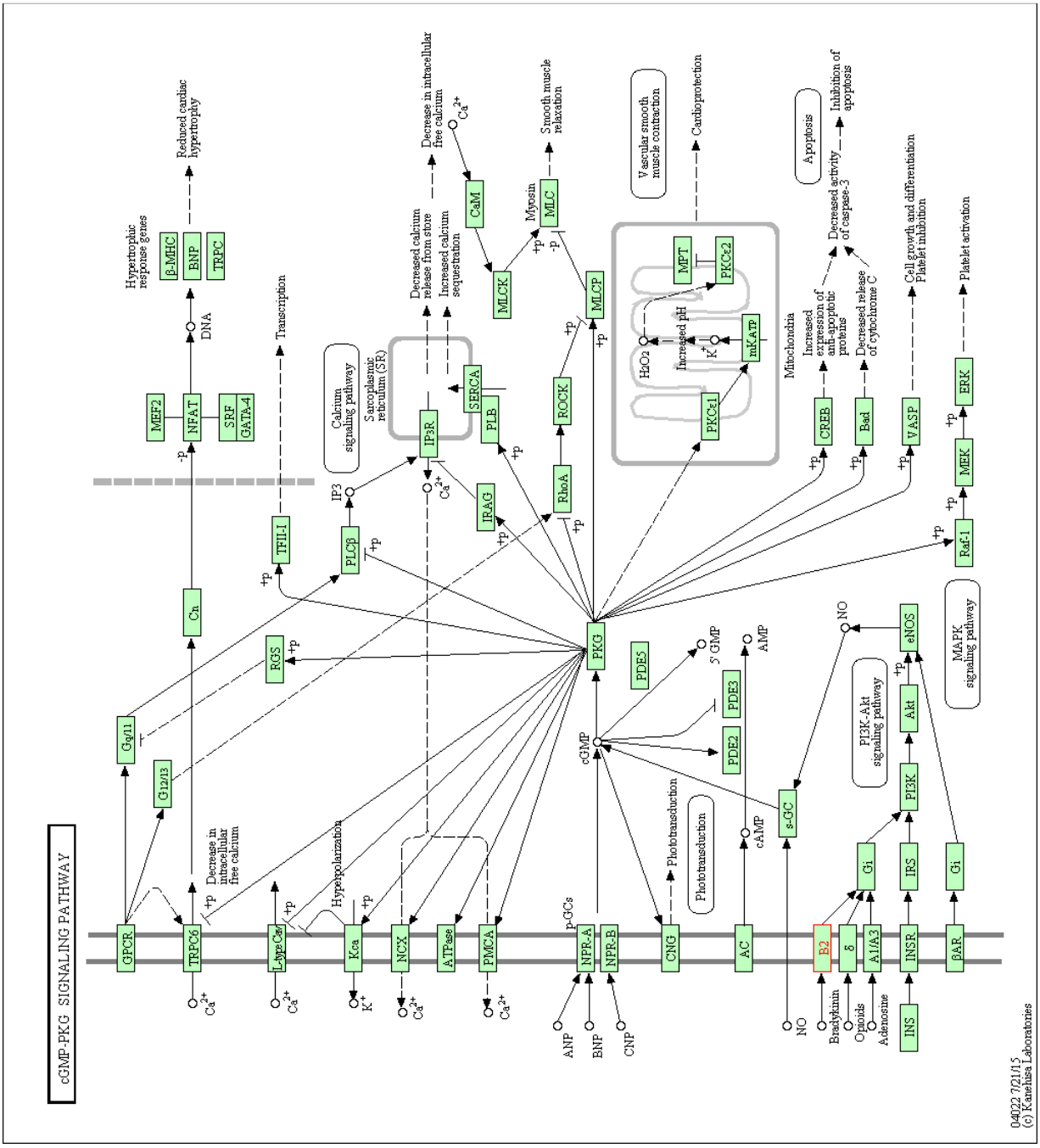


Figure 20[18]: After binding the vasoactive peptide, Bradykinin, downstream sGC (soluble guanylyl cyclase) is activated which raises the level of cGMP within a cell. Higher levels of cGMP lead to an activation of PKG (protein kinase G), which then again activates the MAPK signaling pathway by phosphorylating Raf-1(see image).

5.4.3 Analysis for GABBR2 and GLRA1

GABA and glycine are the main inhibitory neurotransmitters in the dorsal horn (Lamina I and II are GABA dominated, whereas Lamina III and deeper are controlled by GABA and glycine)(Todd & Spike, 1993). The so called gate control theory

[18] http://www.genome.jp/kegg-bin/show_pathway?hsa04022+624

suggests that inhibitory GABAergic and glycinergic interneurons in the spinal cord control the delivery of pain signaling to higher brain areas (Zeilhofer, Wildner, & Yevenes, 2012). Widespread opinions exist that inhibitory neurons play a critical role in pain perception and that dysfunctions in these inhibitory glycenergic and GABAergic systems are pathological mechanisms in many pain conditions including neuropathic pain, allodynia and hyperalgesia (Hwang & Yaksh, 1997; Kuner, 2010; Sandkuhler, 2009; Zeilhofer et al., 2012). Suppressing GABA and glycine neurons led to an enhanced reaction to pain and by agonizing GABA in neuropathic conditions a reversing of hyperalgesia has been observed in many different rat and mice models respectively (Foster et al., 2015; Malan, Mata, & Porreca, 2002). Foster et al showed that through toxin-dependent ablation or silencing of glycine neurons a mechanical and thermal hyperalgesia and itch developed in mice models (Foster et al., 2015).

Mechanisms that underlie the malfunctioning of the inhibitory system such as reduced concentration of GABA and glycine (Moore et al., 2002; Muller, Heinke, & Sandkuhler, 2003; Scholz et al., 2005), reduced responses of postsynaptic glycine receptors (Ahmadi, Lippross, Neuhuber, & Zeilhofer, 2002; Harvey et al., 2004) are present in chronic pain states.

Interestingly the activation of inhibitory neurons can lead to an alleviation of neuropathic pain in certain situations. Peripheral nerve damage leads to higher BDNF concentrations via inflammatory processes, which then again leads to a downregulation of the potassium-chloride co –exporter KCC2 (Coull et al., 2003). The result is a membrane depolarization from -75mV (under non-inflammatory circumstances) to -50mV. The consequence of this pathomechanism is a switch from inhibitory functioning (via hyperpolarization) to an excitatory mechanism in pain processing (Coull et al., 2003).

Consequently, the presence of the minor allele A rs7709656 GLRA1 (p 0.0005) - which was found to be associated with an increased pain perception in midstage KOA- may possibly be due to a disinhibition in the glycenergic system.

GABA-receptor B2 is a metabotropic channel, which is linked to a potassium channel via a G-protein GNB. Its activation through the endogenous inhibitory neurotransmitter GABA leads to a hyperpolarization, hence an inhibitory postsynaptic potential.

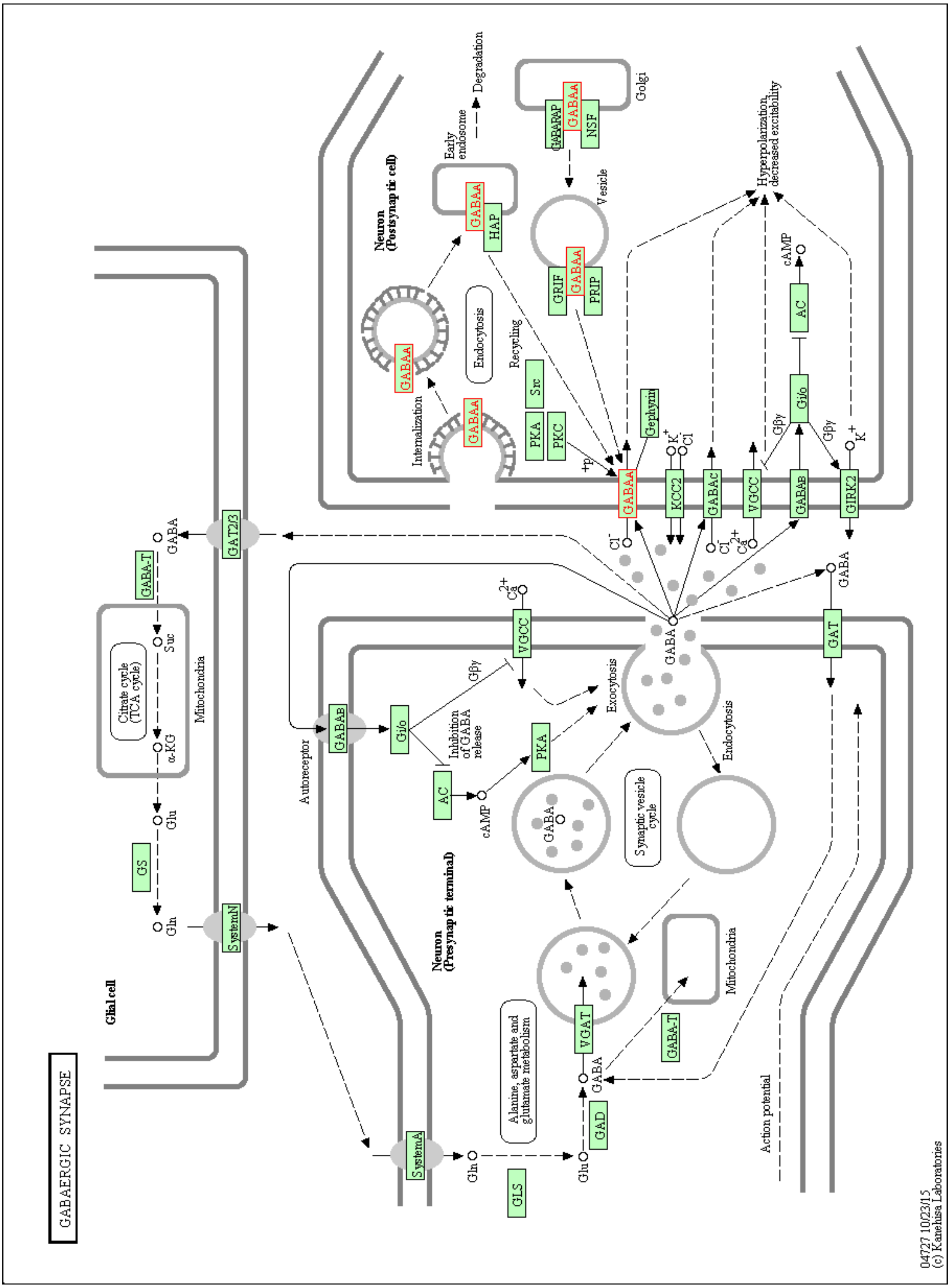


Figure 21[19]: GABA-receptor B is located pre- and postsynaptic. Presynaptic receptors functioning is a negative auto feedback, regulating its own release. Postsynaptic GABA B-receptor subunit 2 heterodimer with GABBR1 (GABA B-receptor subunit 1). Only the

[19] http://www.genome.jp/kegg-bin/show_pathway?hsa04727+2555

heterodimerization allows a functionally active GABA B-receptor, GABBR2 on its own cannot elicit a response downstream(Margeta-Mitrovic, Jan, & Jan, 2000; White JH, 1998).

5.5 MAPK signaling pathway

An observed interface for three genes –TrkA, GABBR2, BDKRB2- was found. Via PathwayLinker it has been observed that the three genes activated the MAPK signaling pathway amongst others.

Mitogen-activated protein kinases (MAPKs) are important for intracellular signal transduction and play critical roles not only in cell differentiation, proliferation but also in regulating neural plasticity and inflammatory responses. (Ji, Gereau, Malcangio, & Strichartz, 2009, p. 1). MAPKs include three major representatives, each standing for its own signaling pathway:

1. Extracellular signal-regulated kinases (ERK1, 2, 5)
2. p38 (p38α, p38β, p38γ, p38δ)
3. c-Jun N-terminal kinase (JNK1, JNK2, JNK3)

Earlier studies have shown, that ERK activation is dependent on nociceptive activity. In normal conditions, ERK is activated by high-threshold mechanical stimulations, this however changes after injury -also in joints- (Ji et al., 2009, p. 4), as it happens in osteoarthritis, posttraumatic and during surgery. Hence, the threshold for pain is reduced. Different mediators, transmitters and pathways can activate ERK downstream in the dorsal horn neurons. The activated ERK leads to translational (via CREB gene expression of proinflammatory mediators and receptors such as and COX-2 and TrkB is upregulated) as well as post-translational changes (recruiting NMDAR, decreasing K+ currents via phosphorylation and hence increasing membrane excitability) both inducing or maintaining central sensitization(Latremoliere & Woolf, 2009). Further a persistent pain condition –with reference to a lowered pain threshold-, like knee osteoarthritis can cause a phosphorylation of MAPKs, thus activating its pathway. Downstream, the synthesis as well as a rapid release of proinflammatory and pronociceptive mediators such as COX2, IL-1β (Kumar et al. , 2003) and TNFα (Boyle et al., 2006) is stimulated. p38 was found to play a central role in inflammation (Boyle et al., 2006; Kumar, Boehm, & Lee, 2003). Ji also proposed and explained how MAPKs take up a crucial part in central sensitization of pain report (Ji et al., 2009).

To sum up, the interaction between our genes and MAPKs may act as an infant attempt to possibly explain how pain is being perceived differently in mid-stage KOA

depending on the presence or absence of certain SNPs, which are again linked with pathways involving MAPKs and possibly causing a sensitization of pain. Nonetheless, it is not seen as necessary to find a single cause to explain all SNPs correlation; a multifactorial origin on genetic level should stay in consideration.

5.6 GPCR (G protein-coupled receptors)

Our found genes encode receptors that all have one characteristic in common: they are G protein-coupled receptors, one of the largest receptor type families with over eight hundred receptors (http://www.ncbi.nlm.nih.gov/biosystems/1269544). After having received an external stimulus -GABA, Bradykinin, Glutamate or Neuropeptides- it comes to a conformational change. GTP replaces GDP and the α subunit dissociates, leaving G$\beta\gamma$ complex. GPCRs are known to have the ability to modulate various pathways, including MAPK signaling (Figure 22) (http://www.ncbi.nlm.nih.gov/biosystems/1269574?Sel=geneid:624&report=Abstract#show=proteins).

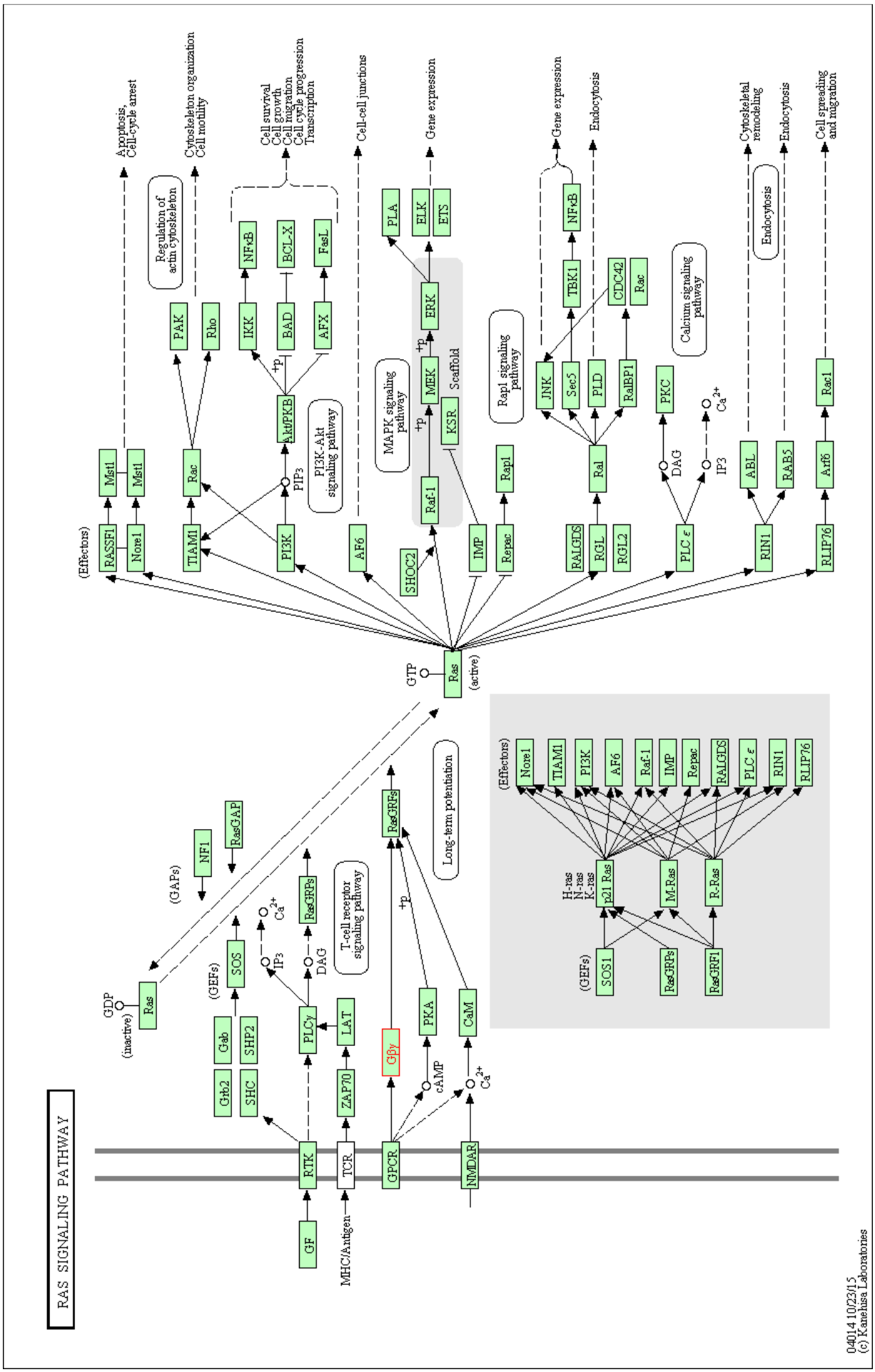


Figure 22: Please see next page for description of figure 22.

Figure 22[20]: GPCR superfamily includes a great variety of receptors. Via Gβγ subunit Ras is activated and leading to an activation of MAPK signaling pathway, which can as mentioned above influence pain perception by central sensitization on molecular levels and immediate release of pronociceptive mediators (Ji, Gereau, Malcangio, & Strichartz, 2009, p. 1).

5.7 Link between TrkA, BDKRB2 and TRP-channels

Nonselective Ca2+-permeable Transient receptor potential channels are built from six transmembrane regions (Huynh, Cohen, & Moiseenkova-Bell, 2014). Six subfamilies exist, including TRPA and TRPV (Bidaux et al., 2015).
TRPA1 (ankyrin 1) receptors are associated with inflammation and nociception. Via PathwayLinker a relation between TrkA and TRP-channel signaling as well as between BDKRB2 and TRP-channel signaling was found (Figure 23).
TRPV1 is well known as the Vanilloid-receptor, functioning as a nociceptor in free nerve endings and can be activated by capsaicin, piperin (pepper), high temperature (>43°C), endogenous cannabinoids, acidic environment (pH<5.2) and proinflammatory substances (Gavenis et al., 2009; Szallasi & Blumberg, 1999).

NGF and BK -two mediators involved in underlying inflammatory pathomechanisms of OA- cause a sensitization and higher expression of TRPV1 in nociceptors via TrkA-dependent and BDKRB2-dependent-pathways –activating PKC downstream, which then phosphorylates TRPV1 at different receptor sites-, as it has been outlined in 1.4.1 (Zhang et al., 2005). This proves that a direct interaction between TrkA, BDKRB2 and TRPV1 exists, which has the potential of altering pain perception.

Shi et al. presented results, which showed that the pain threshold in patients with IBS (irritable bowel syndrome) can be regulated by TRPV1 inhibitors, resulting in a higher threshold (Shi et al., 2015, p. 4857). Derived from this, the idea that TRP channels can regulate pain thresholds generally in chronic pain conditions may also be applied and proven for knee osteoarthritis. As delineated in Chapter 1.4.1, many SNPs have already been associated with pain perception in OA (O'Neill et al., 2012).

To conclude TRPV1, TRPA1, TRPM8 play a role in inflammatory processes and pain mediation in chronic pain patients (Wang et al., 2015, p. 11) and it may be worth exploring more in the field of possible associations and interactions between TrkA, BDKRB2 and TRP-channels.

[20] http://www.genome.jp/kegg-bin/show_pathway?hsa04014+10681

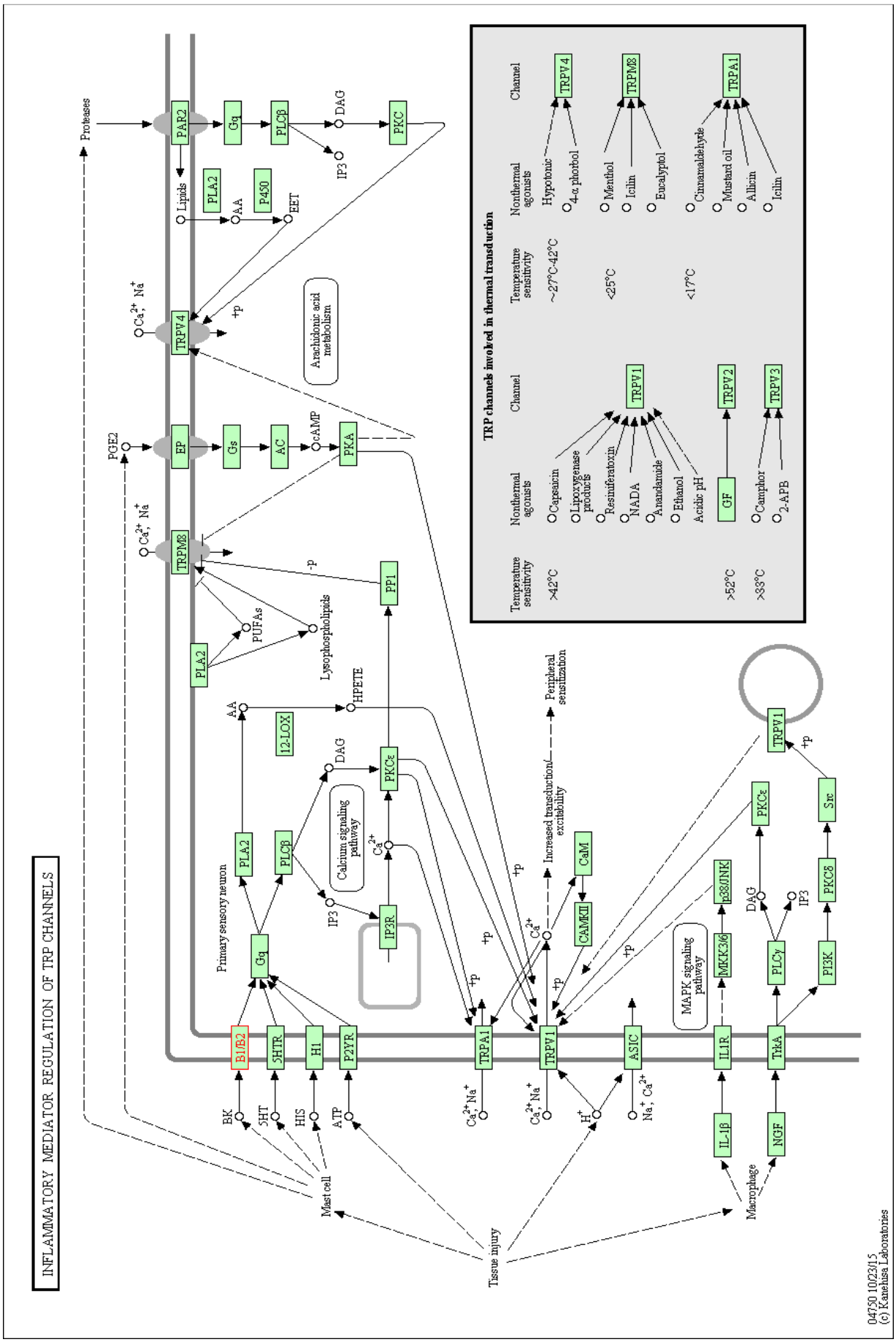


Figure 23: Please see next page for description of figure 23.

Figure 23[21]: Tissue injury and a variety of inflammatory mediators can bind TrkA (NGF) and Bradykinin can lead to the oligomerization of BDKRB1 and 2. This Figure visually illustrates the connection between TrkA and TRPV1/TRPA1 and the relation of BDKRB2 with TRPV1/TRPA1(Katanosaka et al., 2008).

5.8 Future aspects

Many experts are of the opinion that with the momentary trend of increased aging and obesity in the industrialized world, sufficient management of OA becomes of great priority, with some of the goals being the delay of arthroplasty, decelerating the progress of degeneration or even reversing the degenerative process (Fibel, Hillstrom, & Halpern, 2015). Understanding the underlying pathophysiological mechanisms including pain perception in symptomatic KOA patients is crucial for improvement in disease-modifying treatment options.

In this thesis the goal of detecting SNPs non-randomly associated with pain report and treatment response has been fulfilled. In order to validate our observed data replication in an independent set of data should be realized. Another attempt to verify our results can also be achieved through a different study design based on a comparison of pain rating and treatment response between cohorts of patients that present the minor allele of a SNP and patients that present the major allele of that SNP; the independent variable being the allele of a SNP and the dependent measured variable the pain report through standardized questionnaires, WOMAC and VAS e.g.

Another attempt of finding a set of data that acts as a replication to our most significant SNPs or to SNPs which are in strong LD should be tried out, to strengthen the results described in this thesis.

Further, these results give rise to questions such as how does a SNP have an effect upon the encoded protein and via which regulatory mechanisms may it cause a change in pain perception? Quantitative and qualitative measurements of modified proteins may give a greater insight on pathophysiology behind altered pain report.

After having analyzed possible pathways, which proteins and receptors are known to be involved in, the question comes up –with the knowledge that MAPKs activation plays a critical role in induction and maintenance of inflammatory as well as neuropathic pain- on how do modified receptors interact and influence MAPK signaling pathway in pain perception.

[21] http://www.genome.jp/kegg-bin/show_pathway?hsa04750+624

5.9 Possible limitations of this study

A certain reference SNP can be in association to another SNP through linkage disequilibrium, meaning that the observed association between a SNP and a phenotype –altered pain report and altered treatment response in Hypothesis 1 and 2, respectively- is not causal. The actual function of the reference SNP can be inferred based on its position in or near a gene, due to LD between SNPs. For example, a synonymous variants regulation on a genes expression can be explained through linkage disequilibrium. The synonymous variant may show a correlation with another SNP that determines the actual impact on the gene regulation. This non-random association can be exposed using the software mentioned above. This counts as a possible limitation to this thesis.

Due to the population-specific analysis, Caucasians only, impact limitations may arise, and results may not be applicable for other ethnic groups.

The presence of LD between SNPs led to a critical significance niveau of $p < 1.892e^{-5}$, which is in combination with the underpowered study difficult to pass.

Another aspect is that a certain phenotype (altered pain report) can arise from different genotypes, having a complex trait.

Because of the Novelty of this study, gaps of knowledge on SNPs affecting treatment response to ACS exist, which need to be filled by follow-up studies.

6. Summary of results

It can be said the following SNPs for WOMAC rs5224 C>T (BDKRB2), rs7709656 G>A (GLRA1) and for VAS rs1888861 A>G (TrkA) were identified showing a correlation non-coincidentally with alteration in pain phenotype in symptomatic midstage KOA (n=272). An overlap of associated SNPs between baseline pain using either VAS or WOMAC was not observed. The significance threshold was set to $p < 1.892e^{-5}$ after applying the Bonferroni correction. Calculations showed that presence of the minor allele T of rs5224 C>T BDKRB2 and A of rs7709656 G>A GLRA1 correlate with an enhanced pain perception (beta 4.6 – 5.0) in KOA in our dataset. Consequently, presence of these two minor alleles are protective alleles against suffering from painful KOA. The presence of the minor allele G of rs1888861 A>G TrkA is associated with a decrement in pain report with an effect size of -5.2 < beta > -5.0.

Results from regression tests show that the covariates previous history and age do not confound the association between the three SNPs -acting as independent variables- and pain perception –the dependent variable- in KOA in our dataset (n=272). Same SNPs frequently correlate ($9.86e^{-05} < p > 4.99e^{-04}$) with pain report in absence as well as in presence of covariates applied separately and in combination.

Consequently, an enhanced estimation of pain perception through these covariates cannot be given since the most significant SNPs do not alter under various application of covariates.

When BMI was applied as a sole covariate regression analysis found SNP rs3780446 G>A GABBR2 ($p = 0.001653$) to correlate with a decrement of pain report. Rs3780446 G>A being the most significant SNP out of the regression test did not appear to correlate with pain report in tests including other covariates, keeping in mind, that this SNP is markedly below the significance threshold of $p < 1.892e^{-5}$. Test results showed that rs1888861 A>G (TrkA) was not associated with pain report under application of BMI as a sole covariate, as it did with the other covariates age and previous surgery.

Q/Q-plots and the genomic inflation factor, lambda, proposed no systemic deviation, signifying an absence of population stratification, for all regression analyses carried out.

Moreover, a different set of SNPs including rs502434 G>A GRIA3 and rs3782025 T>C HRT3B (when phenotypic WOMAC was used) were singled out

through regression analysis to have an impact ($7.55e^{-05} < p > 2.56e^{-04}$) upon treatment response under ACS, see table 17. When comparing these index SNPs of pain perception and treatment response to ACS, an overlap –which could be explained through this data- was ruled out. SNPs for pain report differed from SNPs involved in treatment response.

A link between 3 SNPs - rs17689135 G>T VPS4B (under WOMAC), rs10011589 T>A GRIA2 (under WOMAC) and rs6490121 A>G NOS1 (under VAS)- and treatment response to HA was detected within our dataset ($0.00052 < p > 0.00096$). Significance of the association does not pass the threshold of $p < 1.892e^{-5}$.

In regression analysis for treatment response to PL one single SNP rs6698337 G>A INADL under application of the phenotypic WOMAC data was found to be associated ($p = 0.00073$), however not passing the Bonferroni threshold, with an increased response to saline injection.

Both Null-Hypotheses[22] for SNP-dependent alteration in pain report and treatment response in symptomatic KOA patients can be discarded and Hypothesis 1 and 2 about a non-coincidental association between SNPs and pain report and treatment response respectively can be accepted.

[22] Null-Hypotheses state that a link between SNPs and pain report and treatment response respectively does not exist.

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Appendix

Annex I: Abbreviations

3’UTR three prime untranslated region

5’UTR five prime untranslated region

AAOS American Academy of Orthopedic Surgeons

ACS autologous conditioned serum

ADAMTS disintegrin and metalloproteinase with thrombospondin motifs

AMPA alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, glutamate receptor

ARE AU-rich element

BDKRB2 bradykinin receptor B2

BDNF brain-derived neurotrophic factor

BK bradykinin

BMI body-mass-index

Bp base pair

CAT catalase

CCP cyclic citrullinated peptide

CEU Western European ancestry

cGMP cyclic guanosine monophosphate

Chr chromosome

CNR1 cannabinoid receptor 1

COMT Catechol-O-Methyltransferase

COX2 cyclooxygenase 2

cPLA2 cytosolic phospholipase A2

CPN1 carboxypeptidase N subunit 1

CRHR1 corticotropin releasing hormone receptor 1

CRP c-reactive protein

DIPJ distal interphalangeal joint

DNA deoxyribonucleic acid

EGFR epidermal growth factor receptor

eQTL expression quantitative trait locus

ERK extracellular signal-regulated kinase

ESR2 estrogen receptor 2

FDA food and drug administration

GABBR2 GABA (gamma aminobutyric acid) receptor B2

GABRA4 gamma-aminobutyric acid type A receptor alpha 4 subunit

GDP guanosine bbisphosphateGLRA1 glycine receptor alpha 1

GNB guanine nucleotide-binding protein (G-protein)

GPA global patient assessment of treatment efficacy

GPCR g protein-coupled receptor

GRIA2 ionotropic glutamate receptor AMPA type subunit 2

GRIA3 ionotropic glutamate receptor AMPA type subunit 3

GTP guanosine triphosphate

GWAS genome wide association studies

HA hyaluronic acid

HIV human immunodeficiency virus

HTR3B 5-hydroxytryptamine receptor 3B

HWE Hardy-Weinberg-Equilibrium

i.v. intravenous

IBS irritable bowel syndrome

IL interleukin

IL-1Ra Interleukin-1 receptor antagonist

IL-1Ra interleukin-1-receptorantagonist

IL10 interleukin 10

INADL alias PATJ, crumbs cell polarity complex component

JNK c-Jun N-terminal kinase

JSN joint space narrowing

KOA knee osteo arthritis

KOA knee osteoarthritis

LD linkage disequilibrium

MAF minor allele frequency

MAOA monoamine oxidase A

MAPK1 mitogen-activated protein kinase 1

MEK mitogen-activated protein kinase kinase

miRNA microRNA

MMP matrix metalloproteinase

MRE microRNA response element

mRNA messengerRNA

NGF nerve growth factor

NMDA N-methyl-D-aspartate

NOS1 nitric oxide synthase 1
NOS3 nitric oxide synthase 3
NRG1 neuregulin 1
NSAID nonsteroidal anti-inflammatory drug
NT neurotrophin
OA osteoarthritis
OARSI Osteoarthritis Research Society International
OPRM1 opioid receptor mu 1
p.o. per os
PACE4 paired amino acid converting enzyme 4
PCSK6 proprotein convertase subtilisin/kexin type 6
PGE prostaglandin E
PGI prostaglandine 1
PKA protein kinase A
PKC protein kinase C
PKG protein kinase G
PL placebo
PNOC prepronociceptin
PRKCE protein kinase C epsilon
PRP platelet rich plasma
PTGS1 prostaglandin-endoperoxide synthase 1
Q/Q-plot Quantile-Quantile-plot
Raf kinase of three serine/threonine-specific protein kinase
Ras a small GTPase
RCT randomized control trials
RNA ribonucleic acid
RoM range of movement
SCN9A sodium voltage-gated channel alpha subunit 9
SF-8 HRQL Short-Form 8 health-related quality of life survey
sGC soluble guanylyl cyclase
SLC6A3 solute carrier family 6 member 3
SNP single nucleotide polymorphism
STAU1 staufen double-stranded RNA binding protein 1
TF transcription factor
TMD temporo-mandibular joint disease
TNF tumor necrosis factor

TrkA tropomyosin receptor kinase A
TRP transient receptor potential cation channel
TRPA1 transient receptor potential cation channel, subfamily A, member 1
TRPM8 transient receptor potential cation channel, subfamily M, member 8
TRPV1 transient receptor potential cation channel, subfamily V, member 1
UNC University of North Carolina
VAS visual analog scale
VEGF vascular endothelial growth factor
VPS4B vascular protein sorting 4 homolog B
WHO World Health Organization
WOMAC western Ontario and McMaster Universities Arthritis Index

Annex II: Labels of tables

Annex III: Labels of figures

Annex IV: WOMAC

The Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC)

Name:_____

Date:_____

Instructions: Please rate the activities in each category according to the following scale of difficulty:

0 = None, 1 = Slight, 2 = Moderate, 3 = Very, 4 = Extremely

Circle one number for each activity

Pain

1. Walking 0 1 2 3 4
2. Stair Climbing 0 1 2 3 4
3. Nocturnal 0 1 2 3 4
4. Rest 0 1 2 3 4
5. Weight bearing 0 1 2 3 4

Stiffness

1. Morning stiffness 0 1 2 3 4
2. Stiffness occurring later in the day 0 1 2 3 4

Physical Function

1. Descending stairs 0 1 2 3 4
2. Ascending stairs 0 1 2 3 4
3. Rising from sitting 0 1 2 3 4
4. Standing 0 1 2 3 4
5. Bending to floor 0 1 2 3 4
6. Walking on flat surface 0 1 2 3 4
7. Getting in / out of car 0 1 2 3 4
8. Going shopping 0 1 2 3 4
9. Putting on socks 0 1 2 3 4
10. Lying in bed 0 1 2 3 4
11. Taking off socks 0 1 2 3 4
12. Rising from bed 0 1 2 3 4

13. Getting in/out of bath 0 1 2 3 4

14. Sitting 0 1 2 3 4

15. Getting on/off toilet 0 1 2 3 4

16. Heavy domestic duties 0 1 2 3 4

17. Light domestic duties 0 1 2 3 4

Total Score: _____ / 96 = _____%

Comments / Interpretation (to be completed by therapist only):

Annex V: Ethics Votum

HEINRICH HEINE UNIVERSITÄT DÜSSELDORF

ETHIKKOMMISSION der Medizinischen Fakultät

ETHIKKOMMISSION der Medizinischen Fakultät der Heinrich-Heine-Universität, Moorenstr. 5, 40225 Düsseldorf

Herrn
Priv.-Doz. Dr. Axel W Baltzer
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27. Jul. 2005

Studiennummer: 1988
Wirksamkeitsstudie zur Behandlung von chronischen, gonarthrose-induzierten Kniegelenksschmerzen mit Orthokin (autologem IL-1Ra): Eine kontrollierte, prospektive und randomisierte Multicenter-Studie

Sehr geehrter Herr Kollege Baltzer,

hiermit bestätigen wir den Erhalt Ihres Schreibens betreffs einer Erweiterung der Nachuntersuchung zur o.g. Studie.
Es bestehen keine ethischen oder rechtlichen Bedenken seitens der Ethikkommission gegen die geplante Erweiterung der Nachuntersuchung. Die Ethikkommission empfiehlt jedoch, in die Patienten-Einverständniserklärung noch folgenden Zusatz (fett gekennzeichnet) aufzunehmen: „Die Weitergabe der Daten erfolgt anonymisiert, das heißt ohne Angaben Ihres Namens, **Ihrer Initialen oder Ihres Geburtsdatums** und wird niemanden außer auf Ihren Wunsch Ihnen selbst zugänglich gemacht". Die Ethikkommission weist weiterhin darauf hin, dass das Datenschutzgesetz verlangt, dass der entsprechende Passus in der Einverständniserklärung im Druck hervorzuheben ist.

Mit freundlichen Grüßen
Ihr

[redacted]

Prof. Dr. Klaus-Dietrich Kröncke
i. A. der Kommission

Zentrum für Molekulare Orthopädie
Gemeinschaftspraxis Königsallee

Orthopädie Neurochirurgie Radiologie

Prof. Dr. med. Peter Wehling
Facharzt für Orthopädie / Sportmedizin
Dr. med. Gregor Godde
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Ethikkommission der
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Düsseldorf, den 05.07.05

Betreff: Studiennummer 1988
Wirksamkeitsstudie zur Behandlung von chronischen, gonarthrose-induzierten Kniegelenksschmerzen mit Orthokin (autologem IL-1Ra): Eine kontrollierte, prospektive und randomisierte Multicenter-Studie

Sehr geehrter, lieber Herr Prof. Lenard,

die Studie zur Evaluierung der Wirkung des autologen Interleukin-I Anatgonisten (Orthokin) wurde zwischenzeitlich erfolgreich abgeschlossen. Sämtliche Hauptzielkriterien und die Nebenzielkriterien wurden bis 6 Monate nach Therapieende erfasst und komplett ausgewertet.

Aufgrund der Wichtigkeit der Analyse der Langzeitwirkung der getesteten Präparate sollten die Patienten in Ergänzung zu unserem Studienprotokoll weitere 6 Monate, insgesamt 1 – 1,5 Jahre nach Therapieende nachuntersucht werden. Dazu sollten die gleichen Instrumente der Datenerfassung und Analyse verwendet werden, die schon bei den 3 vorhergehenden Untersuchungen zur Anwendung kamen.

Zusätzlich sollte zur Optimierung der Erfassung von Langzeit-Nebenwirkungen eine Blutanalyse analog zur Eingangs Blutanalyse erfolgen. Diese sollte ergänzt werden um eine Analyse bestimmter Allele, die nach neuestem Stand der Literatur testen sollen, ob eine individuelle Vorhersage über eine zu erreichende Schmerzreduktion durch die Gabe bestimmter intraartikulärer Substanzen (Orthokin, Hyaluronsäure) möglich ist

(Diatchenko, L, et al. Genetic basis for individual variationsin pain perception and the development of a chronic pain condition. Human Molecular Genetics 2005, Vol. 14, No. 1, 135-143).

Durch die Erweiterung des Umfanges und des Zeitrahmens der Nachuntersuchung ist ein wesentlicher und wichtiger Wissensgewinn in der Medizin zu erwarten. Wir bitten um ein positives Votum der Ethikkommission für die Erweiterung der Nachuntersuchung.

Mit freundlichen, kollegialen Grüßen,
Ihr [redacted]

Priv. Doz. Dr. med. Axel Baltzer
Studienleiter

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