Informed combinations: common genetic variants in MC1R and P2RX7 and their effects on pain phenotypes.

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Abstract

The work that culminates in this thesis has focused on common genetic variants and their impact on pain phenotypes. Two genes were selected for this study – melanocortin-1-encoding *MC1R* and puringergic receptor 7-encoding *P2RX7* – based on their abundance of common nonsynonymous variants and previously published genetic associations with pain. Genetic variability in *MC1R* – all loss-of-function (LOF) variants – is a known contributor to red hair colour, and prior to the publications included in this thesis, it was believed that the same variants contributed to modulating pain sensitivity as red hair, allowing the latter to be an easily identifiable proxy for the former. *P2RX7* is replete with common nonsynonymous variants with an additional advantage for our study in the fact that while most of these variants have a LOF cellular phenotype, others confer a gain-of-function (GOF) on the receptor's cellular phenotype. The original hypothesis tested was: summing like-effect variant alleles may increase the statistical power to detect small effects on a complex phenotype. Thus, in both *MC1R* and *P2RX7* variants were grouped by functional cellular effect into one term, which was tested for association with several pain phenotypes, selected based on previously reported associations and animal model studies. This analysis did not yield better association statistics than individual variant analyses in either gene, suggesting that the additive effect of the entire variant set is not stronger than the effect of the top associated variant and that additional variants likely only contribute noise. However, a number of important discoveries about variant-unifying and variant-discriminating factors resulted from this study. In the gene *MC1R*, nonsynonymous variants that contribute to red hair colour are divided into strong-effect (high-penetrance) and weak-effect (low-penetrance). The haplotype structure and distribution in this genetic locus precludes variants from co-ocurring with observable frequency ($> 0.1\,\%$), which furthermore leads to a flipped direction of effect in single-variant analyses for association between weak-effect variants and red hair. The findings from this study, detailed in Chapter 2, also include evidence for *MC1R* variants carrying the vast majority of the

explanatory power for red hair effects across the entire genome. The second *MC1R* study (Chapter 3) presents evidence for the difference in this gene's regions that harbour variants contributing to red hair versus pain response alterations: while red hair variants lie primarily in the coding region (CDS) of the gene and result in amino acid substitutions, the pain response-associated variants lie primarily in the 5'-untranslated region (5'-UTR). The focus on CDS variants in previous publications may be responsible for opposite directions of effect in red-haired individuals on pain sensitivity. The last study (Chapter 4) is on *P2RX7*'s common ($> 1\,\%$ frequency) nonsynonymous variants, all of which were characterised for cellular functional effect using calcium imaging and patch clamp assays and analysed for association with clinical pain phenotypes, both individually and combined into one term. Contrary to previous publications that reported LOF variant rs7958311 correlating with a reduced risk of chronic neuropathic pain, we found the same variant to confer a higher risk of visceral pain, discovered and validated in two independent cohorts. Combining all LOF variant alleles into one term in the model did not yield better association statistics, which led us to conclude that not all LOF variants are created equal, and despite having the same functional effect as determined by a given cellular functional assay may have different downstream effects on a clinical phenotype. In conclusion, an important summary outcome from the work described in this thesis is the uniqueness of each genetic variant, which merits careful consideration for future association analyses when grouping variants based upon one statistical and/or cellular parameter without performing a comprehensive characterisation of the variant and without considering its surrounding haplotypic structure.

Résumé

Les études qui ont abouti à cette thèse ont porté sur les variants génétiques fréquents et leurs impacts sur les phénotypes de douleur. Deux gènes ont été sélectionnés pour cette étude - le gène *MC1R* codant pour la mélanocortine-1 et le gène *P2RX7* codant pour le récepteur 7 puringergique - en raison de leur abondance en variants non-synonymes et des associations génétiques avec la douleur publiées antérieurement. La variabilité génétique du *MC1R* - tous des variants avec perte de fonction (PDF) - est bien connue pour sa contribution à la couleur des cheveux roux. Dans des études précédant les publications incluses dans cette thèse, il était accepté que ces mêmes variants contribuaient aussi bien à la sensibilité de la douleur qu'aux cheveux roux, ce qui permet à la couleur de cheveux d'être un substitut facilement identifiable pour la douleur. Le *P2RX7* est rempli de variants communs non-synonymes avec un avantage supplémentaire pour notre étude dans le fait que bien que la plupart de ces variants aient un phénotype cellulaire PDF, d'autres confèrent un gain de fonction (GDF) au phénotype cellulaire du récepteur. L'hypothèse originale testée était la suivante: la somme des allèles de variants à effet similaire peut augmenter la puissance statistique de détecter des effets mineurs sur un phénotype complexe. Ainsi, pour chacun des gènes *MC1R* et *P2RX7*, les variants ont été regroupés par effet cellulaire fonctionnel en un seul terme. Ce terme a ensuite été testé pour son association avec plusieurs phénotypes de douleur, sélectionnés sur la base des associations et études sur modèles animaux rapportées précédemment. Cette analyse n'a pas donné de meilleures statistiques d'association que les analyses de variants individuels dans l'un ou l'autre des gènes, ce qui suggère que l'effet additif de l'ensemble des variants n'est pas plus fort que celui du variant associé le plus élevé et que les variants supplémentaires ne contribuent probablement qu'au bruit. Cependant, cette étude a abouti à un certain nombre de découvertes importantes concernant les facteurs unifiants et discriminants des variants. Dans le gène *MC1R*, les variants non-synonymes qui contribuent à la couleur des cheveux roux sont divisés en effets forts (pénétrance élevée) et effets faibles (pénétrance faible). La structure

et la distribution de l'haplotype dans ce locus génétique empêchent les variants de co-apparaître avec une fréquence observable ($> 0.1\,\%$), ce qui conduit en outre à une direction d'effet inversée dans les analyses à un seul variant avec des variants à effet faible pouvant être associées à des cheveux roux. Les résultats de cette étude, détaillés au chapitre 2, incluent également des preuves que des variants du *MC1R* portent la grande majorité du pouvoir explicatif des effets des cheveux roux sur tout le génome. La deuxième étude *MC1R* (chapitre 3) présente des preuves de la différence entre les régions de ce gène qui hébergent des variants contribuant aux cheveux roux par rapport à des altérations de la réponse de la douleur: alors que les variants des cheveux roux se situent principalement dans la région codante (CDS) du gène et entraînent des substitutions d'acides aminés, les variants associés à la réponse à la douleur se situent principalement dans la région non-traduite en 5' (5'-UTR). L'attention sur les variants du CDS dans les publications précédentes peut être responsable des effets opposés observés chez les individus roux sur la sensibilité à la douleur. La dernière étude (chapitre 4) porte sur les variants non-synonymes communs du gène *P2RX7* ($> 1\,\%$ de la fréquence), qui ont tous été caractérisés à l'aide de résultats fonctionnels cellulaires et d'analyses d'imagerie au calcium et de patch-clamp. Ces variants ont ensuite été analysés pour déterminer leur association avec des phénotypes de douleur clinique, d'abord individuellement et ensuite combinés en un seul terme. Contrairement aux publications précédentes qui indiquent une corrélation entre le variant PDF, rs7958311, et un risque réduit de douleur neuropathique chronique, nous avons découvert que le même variant conférait un risque pour la douleur viscéral, découvert et validé dans deux cohortes indépendantes. La combinaison de tous les allèles des variants de PDF en un seul terme du modèle n'a pas donné de meilleures statistiques d'association, ce qui nous a amenés à conclure que toutes les variantes de PDF ne sont pas égales, et qu'elles aient le même effet fonctionnel déterminé par un test fonctionnel cellulaire donné, elles peuvent avoir des effets différents sur un phénotype clinique. En conclusion, l'un des résultats importants résumés des travaux décrits dans cette thèse est le caractère unique de chaque variant génétique, qui mérite une attention particulière pour de futures analyses d'association lors du regroupement de variantes basées sur un paramètre statistique et/ou cellulaire sans procéder à leur caractérisation complète et sans tenir compte de la structure haplotypique environnante.

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Original Contribution

This thesis offers a comprehensive statistical association-based characterisation of genetic variants in melanocortin-1 receptor and purinergic receptor 7 on pain phenotypes. Chapter 2 establishes *MC1R* as the most exclusive explanatory genetic locus responsible for red hair; presents two models – one mRMR-derived parsimonious and the other LASSO-determined comprehensive – for predicting the presence of red hair colour based on *MC1R* variants; and serves as a case study of the statistical flip-flop effect, offering insight into avoiding this pitfall in single-variant genetic association studies. Chapter 3 resolves the incongruity in the previously reported associations for redhaired individuals with pain, showing that most red-hair variants are distinct from pain sensitivity variants, which lie primarily in the regulatory region of the gene. Chapter 4 offers a comprehensive cellular functional characterisation of all common nonsynonymous variants in *P2RX7*, including 5 previously uncharacterised variants, and reports a novel association between the hypofunctional variant rs7958311 and increased risk of clinical visceral pain in two independent cohorts. The thesis also provides a negative answer to the question asked as part of the original hypothesis: whether combining the allele counts of like-effect variants in *MC1R* and *P2RX7* better captures their contribution to a complex phenotype than single-variant association analyses.

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1 Introduction

1.1 Study overview

Pain is an unpleasant sensory and emotional experience brought on by actual or potential tissue damage, as defined by the International Association for the Study of Pain (IASP). Combined with the external environmental risks is interpersonal variability, which makes some individuals susceptible while others resistent to developing chronic pain. Attesting to the growing interest in identifying concrete differences that contribute to this variability, the number of studies of genetic risk factors has been on the rise, reviewed recently by the author of this thesis and her colleagues (1; 2) (also Appendices A and B).

Genetic variants that reach 1 % or higher frequency in the population often do so by offering an evolutionary advantage or by benefitting from a relaxation of the negative selection constraint (3). This implies that their effect is either beneficial or – if detrimental – not sufficiently so to be selected out. With the exception of familial hereditary severe painfull conditions or conditions characterised by insensitivity to pain (1; 2), the genetic variants that impact pain conditions exert minute effects. As such they require large population studies to detect statistical association. While the past decade has seen an increase in large cohort studies, especially through the efforts of international consortia, they continue to be expensive and time-consuming. This is especially true for collecting phenotypes that are part of extensive protocols, such as quantitative sensory testing (QST), that require significant investment of time and energy from participants as well as a willingness to undergo unpleasant or painful procedures.

Another possible approach to optimising statistical power to detect small effects is grouping genetic variants by overall effect. Several axes of division lend themselves well to characterisation. Common, non-lethal genetic variants that do not completely abolish protein function affect their gene's product by either reducing or enhancing its function. In other words, they confer a loss-of-function (LOF) or a

gain-of-function (GOF) effect on the protein's cellular phenotype. This effect can be modulated either directly, by altering its activity in the cell or by reducing protein availability. Variants that affect the protein composition are the proverbial low-hanging fruit. They are the easiest to introduce into model systems and the easiest to test for observable and sometimes quantifiable effects on the protein's function in its pathway(s).

Our original hypothesis was based on the assumption that variants with the same direction of cellular functional effect would have similar effects, if any, on pain phenotypes. Given the binary outcome to the overall expected effect on the clinical phenotype, we considered it plausible that combining variants by direction of effect could increase the statistical power to detect said effect. Every genotype-phenotype relationship has a signal-to-noise ratio, which stands to be diluted by non-contributing variants and magnified by contributing variants, even if their individual effects are very small. With this in mind, an attempt was made to group by direction small, like-effect variants that have shown a pronounced deviation from the wild-type cellular phenotype as established in cell assays to improve association statistics with pain.

The selected genes for this undertaking were *MC1R*, *P2RX7*, and *TRPV1*, all with previous associations with pain phenotypes and all replete with common non-synonymous – amino acid-changing – variants. *MC1R* offered the additional advantage of having an easily observed and quantifiable proxy phenotype, red hair colour, which at the time this study began was believed to be caused by the same genetic variants as alterations in pain response. *P2RX7* and *TRPV1* both had LOF and GOF common nonsynonymous *in vitro* characterised variants.

Combined analysis was attempted, but it did not yield better association statistics than individual variant analysis. Nevertheless, the studies of *MC1R*'s and *P2RX7*'s common nonsynonymous variants led to important discoveries about the genetic landscape of each and their variants' effects on pain phenotypes and have yielded three manuscripts, one published (Chapter 2), one about to be submitted (Chapter 3), and the last in preparation for submission (Chapter 4). The *TRPV1* study has not resulted in a manuscript and is included here as Appendix C.

The gene *MC1R*, which encodes melanocortin-1 receptor, is among the less likely modulators of the pain experience. Variants in this gene were first discovered in connection with their effect on skin and hair pigmentation. Then, genetic mapping, together with association studies driven by anecdotal evidence recounted by clinicians about their patients with red hair, identified the same gene locus as a modulator of pain sensitivity and response to analgesics. Interestingly, the work on

MC1R's role in pain has yielded mixed results: participants with red hair variants have been shown to be both less and more sensitive to painful stimuli. It was therefore our aim to attempt to reconcile previously published findings using a statistical analysis of genotypic and phenotypic data. Resolving the genetic contribution of *MC1R* variants to pain sensitivity and red hair colour started as one project but yielded enough material for two separate manuscripts, which are therefore presented in Chapters 2 and 3. Chapter 2 is a published article analysing the relationship between the *MC1R*'s variants and red hair and revealing its peculiar genotypic substructure. *MC1R* genotypes have a particularly robust statistical association with red hair, an association we exploited to gain a better understanding of its genetic structure. Armed with this knowledge we turned to analysing the much more elusive and intricate relationship between *MC1R* genotypes and pain sensitivity. Chapter 3 is a soon-to-be submitted article that presents new findings on the effect of *MC1R* variants on pain sensitivity.

Chapter 4 presents our findings from the study of *P2RX7*, namely the functional cellular characterisation of previously unassayed nonsynonymous common variants, novel associations with clinical pain phenotypes for a LOF variant rs7958311, with replication in another multiple chronic pain condition cohort. The remainder of the current chapter provides detailed background for *MC1R* and *P2RX7*, and the findings reported in all three manuscripts are summarised in Chapter 5, Conclusion.

1.2 Melanocortin-1 receptor

1.2.1 MC1R's structure and function

Melanocortin-1 receptor, MC1R, is a 7-transmembrane G-protein-coupled receptor expressed in melanocytes. These specialised cells originate in the neural crest and migrate to their final destination, where they differentiate into dendritic, specialised pigment-producers (4). Among the organs that melanocytes populate are the epidermis, hair follicles, and the eye (5), in all of which they produce visible pigment molecules – melanins – contributing to each individual's appearance. The receptor MC1R is an important gate-keeper in the pathway that determines the amount of type of pigment produced and the natural colouration that results. Once activated, MC1R couples to a Gs protein, which turns on adenylyl cyclase and leads to upregulation of cyclic adenosine monophosphate (cAMP). The next step is microphthalmia-

associated transcription factor (MITF)-mediated upregulationg of transcription of tyrosinase, tyrosinase-related proteins 1 and 2 (6) and P protein. The signalling pathway is long and complex, as has been demonstrated in studies of cAMP levels and tyrosinase activity changes post-activation of MC1R: cAMP levels rise within minutes, while tyrosinase activity remains unchanged for many hours (7). Once tyrosinase levels rise and this enzyme engages, with the help of a copper ion it catalyses 3 reactions: tyrosine to DOPA, DOPA to DOPAquinone and DOPAquinone to either black-brown eumelanin or yellow-red pheomelanin in melanosomes, specialised pigment-containing and transporting organelles (8; 9). The type of pigment produced depends on the availability of thiols and intramelanosomal pH (10; 11). The P protein is an integral melanosomal membrane protein that probably stabilises the pigment-production complex (10). The melanin-filled melanosomes migrate along the melanocyte's dendrites from whence they transfer to keratinocytes (12).

The main agonist for MC1R is the α-melanocyte stimulating hormone (α-MSH). In addition to α-MSH, adrenocorticotropic hormone, ACTH, also binds MC1R. Both hormones are cleaved from the same precursor peptide, pro-opioimelanocortin, or POMC and mainly secreted by the intermediate lobe of the pituitary gland (13). There is also evidence that non-POMC-derived molecules bind and activate MC1R (14). An important environmental stimulus for the release of these hormones and consequent MC1R activation is UV irradiation.

Published findings provide evidence for an important damage-control/protective role for α-MSH that manifests in a number of different functions. First, this hormone signals for an upregulation of eumelanin, which absorbs UV radition, converting it to heat. Second, it signals for UV-damaged DNA repair (15). Third, by diverting melanogenic resources away from pheomelanin synthesis it prevents oxidative damage (15). Fourth, it may prevent UV-triggered apoptosis (16).

MC1R activity is regulated by two GPCR kinases, GPK2, which desensitises it and GPK6 which desensitises and also mediates its internalisation (17). The protein residues targeted by these kinases are in the C-terminus of the receptor. Receptor trafficking to the cell membrane is regulated by phosphorylation at the second intracellular loop (18).

1.2.2 The *MC1R* gene and its variants

MC1R was first cloned in 1992 by two groups (19; 20) and mapped to the homologous locus in mice (21). Homologous loci had previously been established as responsible for coat colour in dogs, horses (22) and foxes (23). MC1R is an integral protein, whose main transcript is 317 amino acids long and is encoded by three

exons, although two other protein-encoding transcripts have been catalogued, (24). The first report of *MC1R* genetic variant association with red hair in humans was published in 1995 (25) and the first report of *MC1R* genetic variant association with altered pain sensitivity in mice and humans was published in 2003 (26). Genetic associations with skin cancers (27–30), Parkinson's disease (31), and depression (32) have likewise been reported. With a few exceptions (33), all studies have reported associations for coding-region nonsynonymous and frameshift variants.

1.2.3 Red hair variants

MC1R is a very polymorphic gene, with a high density of nonsynonymous variants. Studies of genetic variation at the single-nucleotide level in *MC1R* began with red hair colour (RHC) association. The model of inheritance best describing the RHC phenotype is recessive: two *MC1R* variant alleles, in either a homozygous (2 minor alleles of the same variant) or compound heterozygous (1 minor alleles from each of 2 different variants) configuration, strongly predict red hair. Not all RHC variant alleles are created equal. During the past 25 years, a consensus has been reached whereby *MC1R* alleles are grouped by penetrance into "R", or high-penetrance, alleles and "r", or low-penetrance alleles. An RR genotype has a 64 % chance of producing red hair; an Rr genotype has a 11 % chance and an rr $<$ 1 % chance (34). R alleles are most prevalent in Central and Northern Europe, and their minor allele frequencies range from 0.0004 (rs201326893) to 0.10 (rs1805007), varying slightly by country (33; 35). The widest frequency-range r allele is rs885479 (R163Q), which is nonexistent in African populations (36) but reaches 78 % in Japanese people (37), 80 % in Han Chinese (38), and 80 % in Tibetans (39). Variant allele fixation is postulated to have resulted from either a positive selection of light skin-conferring alleles (36; 40) or a lack of negative selection (41), both of which would explain geographic distribution of frequencies.

The 3 most common R variants are: rs1805007 (R151C), rs1805008 (R160W) and rs1805009 (D294H). There are also 3 low-frequency coding-region R variants and 3 frameshift variants that have been consistently reported: rs1805006 (D84E), rs11547464 (R142H), rs1110400 (I155T) and rs312262906 (N29insA, merged into rs796296176), rs555179612 (179InsC), and rs201326893 (Y152OCH), respectively. The 3 r variants are: rs1805005 (V60L), rs2228479 (V92M), and rs885479 (R163Q). There have likewise been isolated reports of variants found only in a specific population or associated with a specific condition other than red hair (39; 42). At the same time, some populations far removed geographically from Europe, such as Jamaica, also present European Caucasian *MC1R* R variants (43). While the frameshift vari-

ants are null mutations, the remaining known variants reduce the function of MC1R without abolishing it completely.

During the past twenty years, several research groups have attempted to harness the potential of the strong *MC1R* genotype-RHC phenotype association to determine with appreciable certainty the presence of red hair and developed models for forensic purposes (44–48).

1.2.4 Functional characterisation of *MC1R* variants

A number of studies have characterised the cellular functional effects of *MC1R* variants. The characterisation assays have included mRNA and protein expression, ligand-binding, G-protein coupling, cAMP response, membrane recruitment, receptor desensitisation, and receptor internalisation.

1.2.5 *MC1R* tissue expression

According to the Gene-Tissue Expression (GTEx) project, quantifiable *MC1R* transcript levels have been found in all 48 tested tissues, with enrichment above 20 transcripts per million (TMP) in two brain tissues, cerebellum and cerebellar hemisphere, as well as the pituitary, testis, and thyroid glands, Figure 1.1, (49). The Cancer Genome Anatomy Project also reports *MC1R* cDNA in B-cell, bone, brain, cartilage, colon, embryonic tissue, eye, fetus, gastrointestinal tract, kidney, liver, lymph node, lymphoreticular, muscle, nervous, pancreas, placenta, pooled tissue, skin, stem cell, testis, thymus, uterus, vascular tissue (50).

1.2.6 MC1R pathway in pain

At first glance, pain processing is a seemingly unrelated pathway to melanogenesis. In fact, the two pathways are closely coupled. The chief MC1R agonists, α-MSH and ACTH, share their aptly named pro-opiomelanocortin precursor peptide, POMC, with potent endogenous analgesic molecules: opiod receptor agonists β-endorphin and β-lipotropin (51; 52). The receptors for the melanocortins and opioids are furthermore found in the same tissues (53). Specifically, *MC1R* expression has been found in the periaqueductal grey (PAG) – a brain region with an established role in descending inhibition of the pain response (54; 55), and α-MSH microinjections directly into this region have a reported analgesic effect (56). Numerous animal studies have demonstrated an interaction between the analgesic opioidergic system and anti-analgesic effects of α-MSH and ACTH. With one exception reporting α-MSH microinjections into the periaqueductal grey as inhibitory of the pain response, (56), this hormone has been consistently demonstrated to have proalgesic effects in rodent models (57–59) and in rabbit (60). ACTH is also linked to hyperalgesia in rat

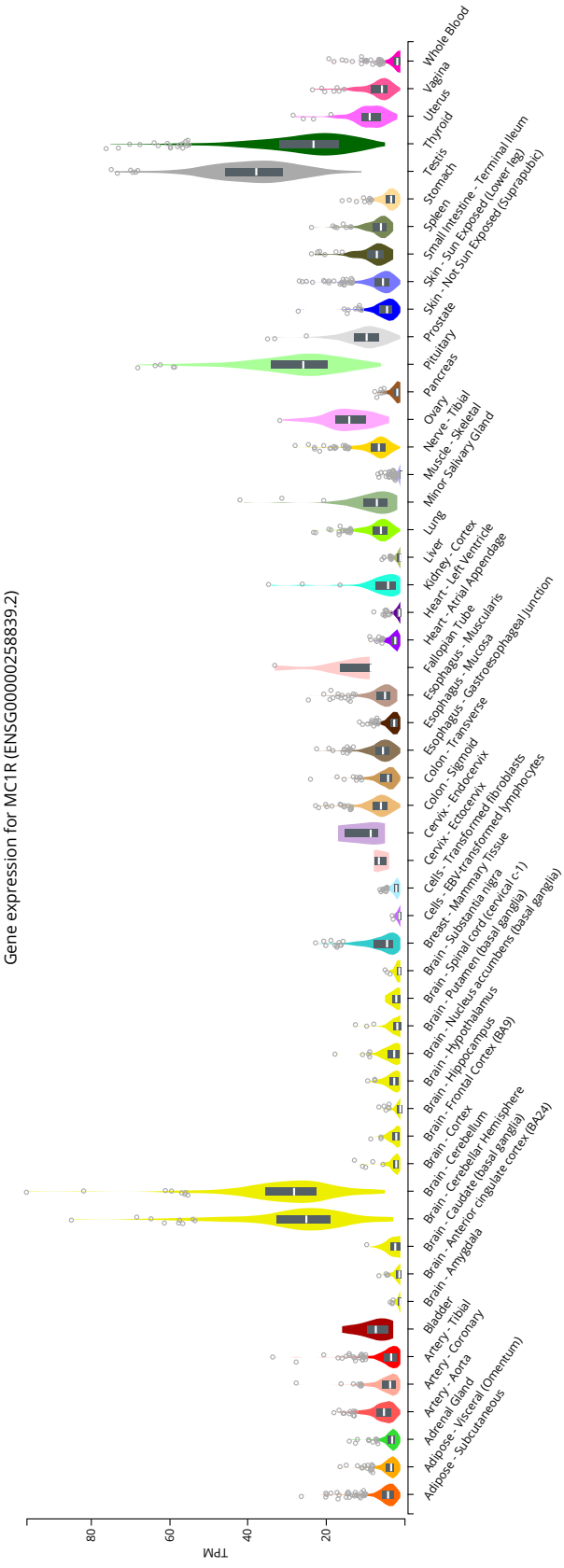

Figure 1.1: *MC1R* expression levels in GTEx tissues.

(61) and rabbit (60) and appears to modulate nociception by interacting with the endogenous opioid system (61). Their mode of action is believed to be antagonism of opioid receptors (62). Although α-MSH and ACTH also activate MC4R (63), whose preferential distribution in the brain and spinal cord (13) makes it the more likely melanocortin receptor to be involved with nociception, selective targetting of MC1R has implicated specifically this receptor in nociception. A study targetting the role of MC1R in nociception found reduced sex-specific inflammation(formalin)-induced hyperalgesia and allodynia in female mice with either an *Mc1r*-null genotype or when administered an MC1R antagonist (58). The authors further tested noxious heat and capsaicin response in the mice and found the same effects, which led them to suggest an interaction between MC1R and transient receptor potential vanilloid 1, TRPV1. This study also reported no effect of either genetic disruption or antagonism of MC1R on neuropathic pain.

The *MC1R* cell type expression profile suggests a role for its mediation of pain accompanying inflammation. The receptor has been noted in a number of immune system cells, including macrophages, monocytes, neutrophils, endothelial cells, and astrocytes, and α-MSH has been shown to downregulate proinflammatory cytokines, such as interleukin-1α, interleukin-1β, interleukin-6 and TNF-α, as well as the nociception-mediating neurotransmitter nitric oxide, while upregulating anti-inflammatory interleukin-10 and interleukin-8 (64).

Furthermore, α-MSH and ACTH are released from the pituitary gland after POMC is upregulated under conditions of stress (65). An early study in a formalin-induced chronic pain mouse model showed that immobilisation stress produced analgesia, which was abolished by hypophysectomy (removal of the pituitary gland) (66). The authors proposed that POMC-derived opioid receptor agonists that would normally engage in pain inhibition were not available. Interestingly, an attempt at rescuing the analgesia via an ACTH injection did not produce the desired effect, leaving them to conclude that other POMC-derived ligands were responsible for pain downregulation under stress and chronic pain conditions. While this places the centre of action upstream of MC1R involvement, a feedback loop condition, under which low MC1R activity signals for increased levels of POMC is an attractive and plausible explanation.

Human studies have been limited to genotype-phenotype and phenotype-phenotype association, in which the phenotype red hair was used as the proxy for *MC1R* variants and tested for association with pain sensitivity. The first association for *MC1R* variants with pain sensivity in humans was published in 2003 by Mogil and colleagues (26). This work reported that red-haired women required less

of a $\varkappa$-opioid analgesic under experimental painful ischemic and heat pain protocols. Two years later, the same group published another study reporting a non-sex specific favourable response in red-haired individuals to a mu-opioid analgesic under experimental painful electrical stimuli protocol (67). Two contemporary publications offered contradictory findings, both reporting red-haired women requiring more anesthesia for experimental pain protocols (68; 69), and in the case of the latter publication also a higher baseline sensitivity to hot and cold stimuli. In another study published 6 years later, women with red hair were found to be less sensitive to topical capsaicin-induced pin-prick hyperalgesia (but not heat or pressure hyperalgesia) (70). The type of pain response tested – mechanical stimuli applied to an existing an area with capsaicin-induced painful inflammation – aimed to assess susceptibility to central sensitisation.

There are also human studies suggesting that MC1R-mediated effects on pain takes place via the stress-processing pathway. In support of this is a report about *MC1R* red hair variant-carriers having more anxiety about dental procedures and pain-related fear (71). A similar study showed that *MC1R* variants responsible for red hair were associated with higher levels of dental anxiety, but not anesthetic efficacy of the applied nerve block (53). The main source of α-MSH's precursor POMC is the pituitary gland, a titular component of the hypothalamic-pituitary-adrenal axis, whose role in anxiety mediation is well established (72).

1.3 Purinergic receptor-7

1.3.1 P2X7R structure and function

P2X7R is an immune-system responder activated by high concentrations of ATP (minimum 100 μM). Its response is bimodal: initial ligand binding opens a channel that nonselectively admits calcium and other cations. Prolonged contact with ATP results in the opening of a large plasma pore, the exact provenance of which has been under debate. One theory is that the P2X7R channel itself widens to admit large molecules (73), while another posits that extended ATP stimulation causes P2X7R to bind another plasma membrane protein, pannexin-1 (PANX1), which opens into a large pore (74; 75). It is furthermore possible that both proposed mechanisms could be correct, if one considers that different isoforms of P2RX7 function in distinct ways (76–78).

A significant amount of published research has accumulated characterising P2X7R's involvement in the pro-inflammatory response to injury or pathogenic invasion. Compromised or apoptotic cells release ATP into the extracellular space, where it reaches high concentrations and binds the low-affinity P2X7 receptor, opening a cation channel. Subsequent calcium entry initiates a cascade to assemble an inflammasome, which brings to maturity caspase-1 (CASP1), the main protease responsible for cleaving pro-interleukin-1β into its active form (79). Once released, IL-1β binds interleukin-1 receptors and signals for the onset of inflammation (79). Interleukin-6 and tumour necrosis factor(TNF)-α are also released by the cell following P2X7R activation (80).

Interestingly, exposure to ATP induces a biphasic calcium response, and it has been proposed that P2X7R recruits other cation channels upon activation (81). It has also been suggested that the pannexin-1 pore serves as a conduit for ATP out of the cell (82; 83). Given that this conduit is opened by calcium waves caused by ATP-stimulated P2X7R (84), the purine appears to participate in a self-perpetuating cycle of activation. Ferrari et al. have proposed that ATP is a constitutive signal of danger present throughout all tissues; it is its increase to P2X7R-activating concentrations, however, that drives a full-scale inflammatory response (85). Surprisingly, low levels of ATP prove to be anti-inflammatory, which may indicate that other purinergic receptors with a higher sensitivity for ATP mediate an anti-inflammatory response and that the decision to inflame or not to inflame is made down-stream of extracellular ATP — likely by P2RX7 (86).

Differences between purinoceptor 7 and other purinergic receptors underscore its unique functionality. P2X7R differs from other members of the P2X family in: 1) its low sensitivity to ATP; 2) its long C- terminus with protein and lipid interaction sites and a cysteine-rich stretch of 18 amino acids 3) its ligand-gated activity, dependent on absence or reduction of divalent cations from the surrounding medium 4) its two modes of operation: through a non-selective cation channel and through a large molecule-admitting pore (74). With many sites of protein-protein, protein-ligand, and protein-cofactor interactions, P2R7X presents as a model of a highly complex and versatile agent of the body's defense system that can be tweaked to perform a wide variety of cellular functions.

1.3.2 The *P2RX7* gene and its variants

The P2X7 receptor is encoded by *P2RX7*, a highly polymorphic gene with over 130 single nucleotide variants. A number of common variants have been found and examined in genetic association studies as well as functional assays. Among these, 2 GOF

variants have been reported – rs208294 and rs17525809 (85) – and 7 LOF variants – rs7958311, rs2230911, rs28360447, rs1653624, rs7958316, rs28360457, rs3751143 (87–89). The reported direction of effect for variants rs2230912 and rs1718119 (85) has been mixed, with different cellular phenotypes being shown in different studies (90–93). It is likely that variants in different parts of the receptor affect different cellular functions, which may lead to different pathologies. In particular, evidence has been provided for the pore-opening and the variants that affect it to mediate pain processing (94).

1.3.3 Functional characterisaiton of *P2RX7* variants

1.3.4 *P2RX7* tissue expression

According to the Gene-Tissue Expression (GTEx) project, quantifiable *P2RX7* transcript levels have been found in almost all tested tissues, with substantial presence in the spinal cord and other central nervous system tissues, Figure 1.2.

1.3.5 P2X7R in pain: animal studies

The activity of P2RX7 has been demonstrated in a number of animal model studies to correlate with increased pain. One of the earliest reports on the role of P2X7R in pain processing comes from Dell'Antonio et al., who tested oxidized ATP, a P2X7R inhibitor, on an acute inflammatory pain rat model and discovered an anti-hyperalgesic response (95). Shortly thereafter, Chessell et al. used a genetic/behavioural approach to uncover that P2X7R-knockout neuropathic mice displayed no hypersensitivity under induced inflammatory pain conditions (96). Mice with partial nerve ligation for the neuropathic model were tested for thermal sensitivity, measured as a function of paw withdrawal times on a hot plate, and tactile allodynia (sensitivity to innocuous touch), using Von Frey hair filaments. Their results indicated that P2X7R was a necessary and non-redundant component of the nociceptive processing system, as did another study in a neuropathic pain mouse model by Sorge and colleagues (94). Additionally, pharmacological blocking of this receptor was assessed by Chessell et al. and a number of others, (97–99), all of whom found the result to be reduction in hyperalgesia and/or inflammation. Huang et al. furthermore found activation in microglia and astrocytes and upregulated expression of P2X7R in bone cancer rats, whose pain and whose levels of 5-HT and Fos expression were reduced in the spinal cord by RNAi knock-down of the purinoceptor (100).

1.3 Purinergic receptor-7

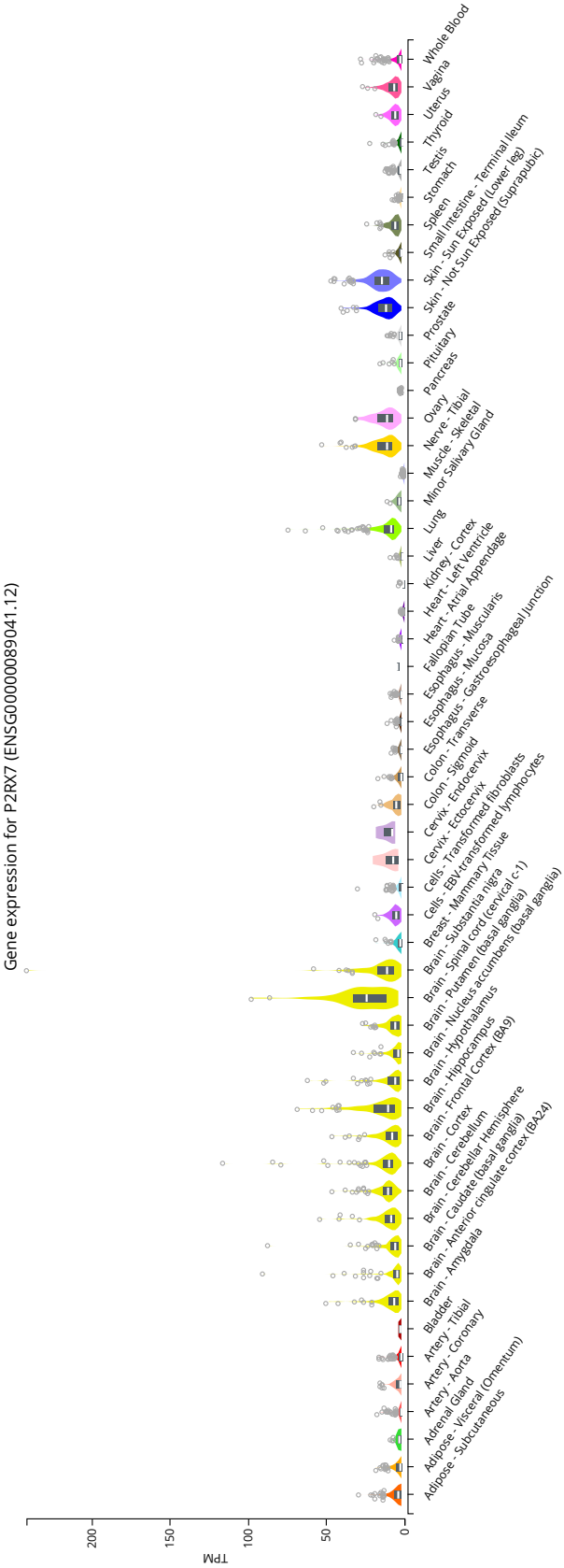

Figure 1.2: *P2RX7* expression levels in GTEx tissues.

1.3.6 *P2RX7* variants in human pain conditions

Despite the high number of common nonsynonymous variants in the *P2RX7* locus, genetic studies have shown only 3 variants to be associated with pain. GOF variant rs208294 has been reported to be associated with increased pain in post-mastectomy patients while LOF variant rs7958311 has been reported associated with reduced pain in the same cohort and in osteoarthritis patients (94). Variants rs208294 and rs1718119 (unclear cellular phenotype) have likewise been reported to be associated with higher pain scores in diabetic neuropathic patients (93) and rs7958311 with experimental cold pain and with clinical pain (7-day post-operative pain intensity and multisite chronic pain) (101).

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2

A study in scarlet: *MC1R* as the main predictor of red hair and exemplar of the flip-flop effect.

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Author contributions: K. Z.-L. conceived the idea about haplotypic structure resulting in a flipped direction of effect for r variants, did single-variant and LD analyses, and wrote the manuscript. Prediction modeling, consisting of mRMR scoring, GLM and LASSO, was done by R.N.L., who also wrote corresponding methods sections. D.Z. provided text for descriptions of the flip-flop effect. M.P. prepared phenotype files and ran preliminary association analyses. A.B. ran preliminary haplotype analyses. S.G. advised on population genetics. L.D. supervised the development of ideas, reviewed and edited the manuscript.

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Genetic variation in melanocortin-1 receptor (*MC1R*) is a known contributor to disease-free red hair in humans. Three loss-of-function single-nucleotide variants (rs1805007, rs1805008, and rs1805009) have been established as strongly correlated with red hair. The contribution of other loss-of-function *MC1R* variants (in particular, rs1805005, rs2228479, and rs885479)

and the extent to which other genetic loci are involved in red hair colour is less well understood. Here, we used the UK Biobank cohort to capture a comprehensive list of *MC1R* variants contributing to red hair colour. We report a correlation with red hair for both strong-effect variants (rs1805007, rs1805008, and rs1805009) and weak-effect variants (rs1805005, rs2228479, and rs885479) and show that their coefficients differ by two orders of magnitude. On the haplotype level, both strong- and weak-effect variants contribute to the red hair phenotype, but when considered individually, weak-effect variants show a reverse, negative association with red hair. The reversal of association direction in the single-variant analysis is facilitated by a distinguishing structure of *MC1R*, in which loss-of-function variants are never found to co-occur on the same haplotype. The other previously reported hair colour genes' variants do not substantially improve the *MC1R* red hair colour predictive model. Our best model for predicting red versus other hair colours yields an unparalleled area under the receiver operating characteristic curve of 0.96 using only *MC1R* variants. In summary, we present a comprehensive statistically-derived characterisation of the role of *MC1R* variants in red hair colour and offer a powerful, economical, and parsimonious model that achieves unsurpassed performance.

2.1 Introduction

Melanocortin-1 receptor (*MC1R*) is a seven transmembrane G protein-coupled receptor, encoded by the gene *MC1R* on 16q24.3 (1). Endogenously activated by the melanocyte stimulating hormone, α-MSH, and the adrenocorticotropic hormone, ACTH, this receptor is a critical component of skin and hair pigment biosynthesis. Upon ligand binding, it signals for the activation of adenylyl cyclase, which increases cyclic adenosine monophosphate (cAMP) production and leads to the assembly of a multi-protein complex, stabilised by the P gene protein (2). The multi-protein complex is directly responsible for the conversion of precursor DOPAquinone to eumelanin, otherwise preferentially converted to phaeomelanin (3; 4). The eumelanin-to-phaeomelanin ratio determines skin and hair colour in humans and coat colour in other mammals (4–7).

A connection between non-synonymous polymorphisms in (MC1R)-encoding gene and human hair colour was first established by Valverde and colleagues in 1995 (6). Since then, many studies have been done to confirm these associations,

refine the effects of individual variants, and dissect the mechanisms whereby these variants modulate human pigmentation. In the compendium of literature on *MC1R* variants and human pigmentation published during the last 22 years several conclusions are apparent: 1. all human *MC1R* functionally-characterised nonsynonymous variants confer a loss of function (of varying degrees); 2. the variants with the highest functional effect demonstrated *in vitro* confer red hair colour and sun-sensitive skin with poor tanning ability; 3. these traits are expressed on different genetic backgrounds and skin pigmentation profiles indigenous to different geographic locations; 4. the model of inheritance for these pigmentation phenotypes is recessive with a dose-dependent effect, i.e. simple heterozygotes may exhibit a shade of red that lies between wild-type and variant homozygote or compound heterozygote extremes (8; 9); 5. *MC1R* variants are necessary to express a disease-free red hair phenotype (10).

Although *MC1R* is an unusually polymorphic gene (11), only two of its high-penetrance variants, rs1805007 and rs1805008 (amino acid changes R151C and R160W, respectively) are prevalent across all populations studied for red hair (12–15). Additionally, rs1805009 (D294H) has a noticeable presence in the British Isles and in the Netherlands (6; 12; 16). Aside from these three variants, 6 rare high-penetrance variants have been observed in various populations: rs1805006 (D84E), rs11547464 (R142H), rs1110400 (I155T), rs312262906 (N29insA, merged into rs796296176), rs555179612 (179InsC), and rs201326893 (Y152OCH) (8; 16; 17). These 9 variant alleles have been nicknamed "RHC" or "R" alleles to denote their high penetrance and strong association with the red hair colour (13), and individuals who have at least two of these alleles (either in a homozygous or compound heterozygous configuration) exhibit pure red hair in up to 96 % of cases (18). Furthermore, three nonsynonymous low-penetrance common *MC1R* variants – rs1805005, rs2228479, and rs885479 (V60L, V92M, and R163Q, respectively) – have been reported and designated "r" alleles (19). These vary in their minor allele frequency across different populations and have been found to have a correlation with red hair ranging from weak (19–21) to none (8; 13). Among these, rs1805005 has been reported to be weakly associated with blonde hair (22). All 12 variants are marked in the *MC1R* schematic, Figure 2.1.

MC1R variant distribution differs widely between different parts of the world. The highest frequency of R alleles is observed in Northern Europe, whereas in more sun-exposed geographic regions, where red-haired individuals are much rarer in the native populations, R alleles are also quite rare. Red-haired carriers of R alleles have also been reported among European descendants in South Africa (23) and Australia

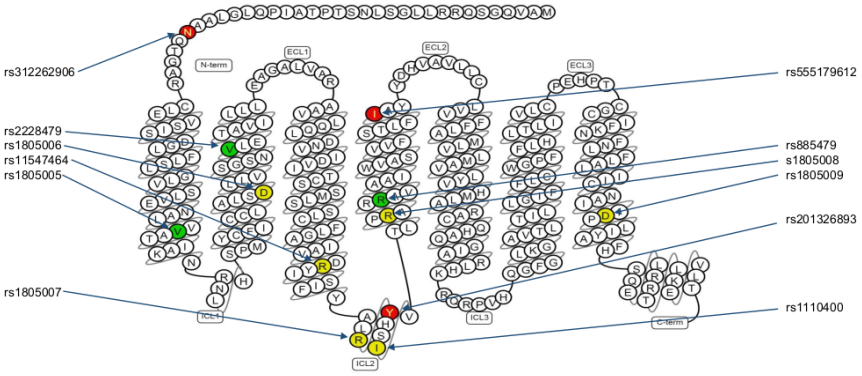


Figure 2.1: Previously reported variants in *MC1R* associated with red hair. High penetrance R variants are in yellow: D84E (rs1805006), R142H (rs11547464), R151C (rs1805007), I155T (rs1110400), R160W (rs1805008), and D294H (rs1805009), and low-penetrance r variants are in green: V60L (rs1805005), V92M (rs2228479), R163Q (Rs885479). Frameshift variants N29insA (rs796296176), 179insC (rs555179612), and Y152OCH are in red. Frameshift variants N29insA (rs796296176), 179insC (rs555179612), and Y152OCH (rs201326893) are in red. Image courtesy of GPCRdb.org.

(19), darker-skinned Southern Europeans (15), a darker-skinned Mongolian family (24), and black Jamaicans (14). On the other hand, r variants rs2228479 and rs885479, which are not known to have a strong effect on red hair, appear to be highly prevalent, reaching frequencies up to 73 % in East Asia (25; 26).

Several studies have reported cellular assays showing functional impairment for versions of *MC1R* carrying the common six variants: rs1805007, rs1805008, rs1805009, rs1805005, rs2228479, and rs885479 (27; 28). While these studies diverge on the extent of some functional effects, there is consensus on the receptor's signalling for cAMP production, in which the first 3 (R) variants confer considerable impairment and the latter 3 (r) show milder effects (28–30). The variants have also been classified *in silico* according to their cross-species conservation (SIFT) and according to their predicted structural alterations (PolyPhen) as tolerant and intolerant, with R alleles predictably falling in the latter category (31; 32). The only two known complete loss-of-function (or null) variants are rs796296176 and rs555179612 (17).

Here, we sought to exploit the statistical power of the 500,000 individuals in the U.K. Biobank (33) to answer several outstanding questions regarding red hair genetics: 1. whether nonsynonymous (amino-acid-changing) coding-region *MC1R* variants are the primary effectors of the red hair phenotype; 2. whether r variants have any quantifiable contribution to this phenotype; 3. whether variants in other

Table 2.1: Minimum redundancy maximum relevance (mRMR)-ranked *MC1R* variants in red versus dark hair colour prediction

Rank	Variant	Function	MAF	Info score	Penetrance	mRMR score
1	rs1805007	missense	1.03e-2	1.28e-1	R	1.28e-1
2	rs1805008	missense	8.3e-2	3.58e-2	R	3.58e-2
3	rs1805009	missense	2.8e-2	2.17e-2	R	2.17e-2
4	rs2228479	missense	9.7e-2	8.4e-3	r	8.4e-3
5	rs312262906	frameshift	5.5e-3	7.1-e3	R	7.1-e3
6	rs11547464	missense	7.2e-3	4.4e-3	R	4.4e-3
7	rs885479	missense	4.6e-2	4.1e-3	r	4.1e-3
8	rs1805006	missense	1.22e-2	3.56e-3	R	3.56e-3
9	rs555179612	frameshift	1.93e-3	3.25e-3	R	3.25e-3
10	rs1805005	missense	1.11e-1	2.45e-3	r	2.45e-3

The designations R, high-penetrance, and r, low-penetrance, are based on previously reported associations with red hair. Info score is a measure of imputation quality.

genes have any contribution to red hair beyond *MC1R*; 4. whether *MC1R* variants have an effect on other hair colours. In addition, we developed a high-powered statistical model trained on this dataset using only *MC1R* variants as predictors.

2.2 Results

2.2.1 Association analysis for *MC1R* variants and red hair

First, we sought to confirm the previously reported variants as the primary effectors of the red hair phenotype. We used the mRMR algorithm to determine whether the explanatory power for this phenotype (red versus dark hair) lay primarily in the coding region and with nonsynonymous variants. Although the algorithm was run on all imputed *MC1R* variants, the 10 top variants in the output, Table 2.1, were indeed nonsynonymous coding region variants, 8 missense (rs1805007, rs1805008, rs1805009, rs2228479, rs11547464, rs885479, rs1805006, and rs1805005) and 2 frameshift (rs796296176 and rs555179612), Figure 2.1. Seven of these variants (rs1805007, rs1805008, rs1805009, rs796296176, rs11547464, rs1805006, and rs555179612) have been previously reported as high-penetrance (R), and the remaining 3 (rs2228479, rs885479, and rs1805005) as low-penetrance (r). Thus, all the known common r variants and 7 of 9 known R variants were selected by our mRMR algorithm among the top discriminators of red hair.

Second, we passed each of these variants and the two other previously-reported R alleles (rs201326893 and rs1110400) using a GLM as an individual determiner of the binary phenotype – red versus dark hair – to ascertain the direction of their effect on red hair. The results are presented in Table 2.2. In confirmation of the well-established recessive model of inheritance for red hair, the effect size of R variants is substantially larger for the recessive model than for the additive model. While previous publications have disagreed on the effect of common r variants (rs1805005, rs2228479, and rs885479), with most reporting them as either silent or weakly associated with red hair, our results show for r alleles – surprisingly – a negative, significant correlation with red hair (and therefore positive correlation with dark hair), and for R alleles, the expected positive, significant correlation with red hair.

2.2.2 Assessment of independent effects of r alleles.

Next we tested if the haplotypic structure of *MC1R* could be a possible explanation for the negative correlation between r alleles and red hair in the above analysis. The variants in the coding region have been reported to exhibit almost no pairwise linkage disequilibrium (LD, as measured by r-squared) in individuals with European ancestry (34). To reproduce and explore this finding, we visualised the LD pattern with variants included in Table 2.2 in Haploview, Figure 2.2. Of note, frameshift variants rs796296176, rs555179612, and rs201326893 had insufficient minor allele frequencies (5.5e-3, 1.93e-3, and 2.56e-4) to be included in linkage disequilibrium determination by Haploview. While, consistently with previous reports, we did not observe any correlation as measured by r-squared (diamond colour in the LD plot, Figure 2.2), we did see strong LD between all included variants using the D' measure (value inside the diamond, Figure 2.2), which denotes the pairwise LD coefficient with the range unaffected by the difference in minor allele frequencies (r can range from -1 to 1 only when both minor allele frequencies are the same). Our results indicate that given all existing >1 %-frequent haplotypes in this region (Table 2.3) no 2 variants' minor alleles co-occur on the same haplotype/chromosome. It follows that having any one variant allele precludes the possibility of having another one on the same chromosome. By extension, being heterozygous for an r variant allele means at most being heterozygous for one R variant allele; and being homozygous for an r effectively nullifies the chance of having any R variants and therefore drastically reduces the chance of having red hair. This finding suggests that the negative association coefficient for r variants may be indicative of the absence of R minor alleles rather than of their own direct effect on red or dark hair.

In this branch of investigation, it remained to answer the question whether the

Table 2.2: GLM output for single-variant associations with red versus dark hair

Variant	MAF	Info score	Penetrance	Additive model Effect (OR)	*P* value	Recessive model Effect (OR)	*P* value
rs312262906	5.5e-3	0.82	R	9.95	<2e-16	NA	NA
rs1805005	1.11e-1	1	r	0.345	<2e-16	0.1036	<2e-16
rs1805006	1.22e-2	1	R	3.48	<2e-16	10.63	2.81e-9
rs2228479	9.7e-2	1	r	0.109 1.09e-1	<2e-16	0.03357	<2e-16
rs11547464	7.2e-3	1	R	4.7	<2e-16	346.7	2.30e-8
rs1805007	1.03e-1	1	R	12.7	<2e-16	272.1	0
rs1805008	8.3e-2	1	R	5.12	<2e-16	35.57	0
rs885479	4.6e-2	1	r	0.163 1.63e-	<2e-16	0.100	1.99e-8
rs1805009	2.78e-2	0.93	R	6.66	<2e-16	648.1	<2e-16
rs555179612	1.93e-3	1	R	10.56	<2e-16	NA	NA
rs201326893	2.56e-4	1	R	10.12	<2e-16	NA	NA
rs1110400	1.08e-2	0.98	R	1.32	4.96e-9	NA	NA

The designations R, high-penetrance, and r, low-penetrance, are based on previously reported associations with red hair. Effect size, here, OR > 1 denotes a positive association with red hair, and OR < 1 denotes a negative association with red hair. Association statistics are listed as NA, not available, for the recessive model for rare frameshift variants because there were no individuals homozygous for the minor allele at these variants. Info score is a measure of imputation quality.

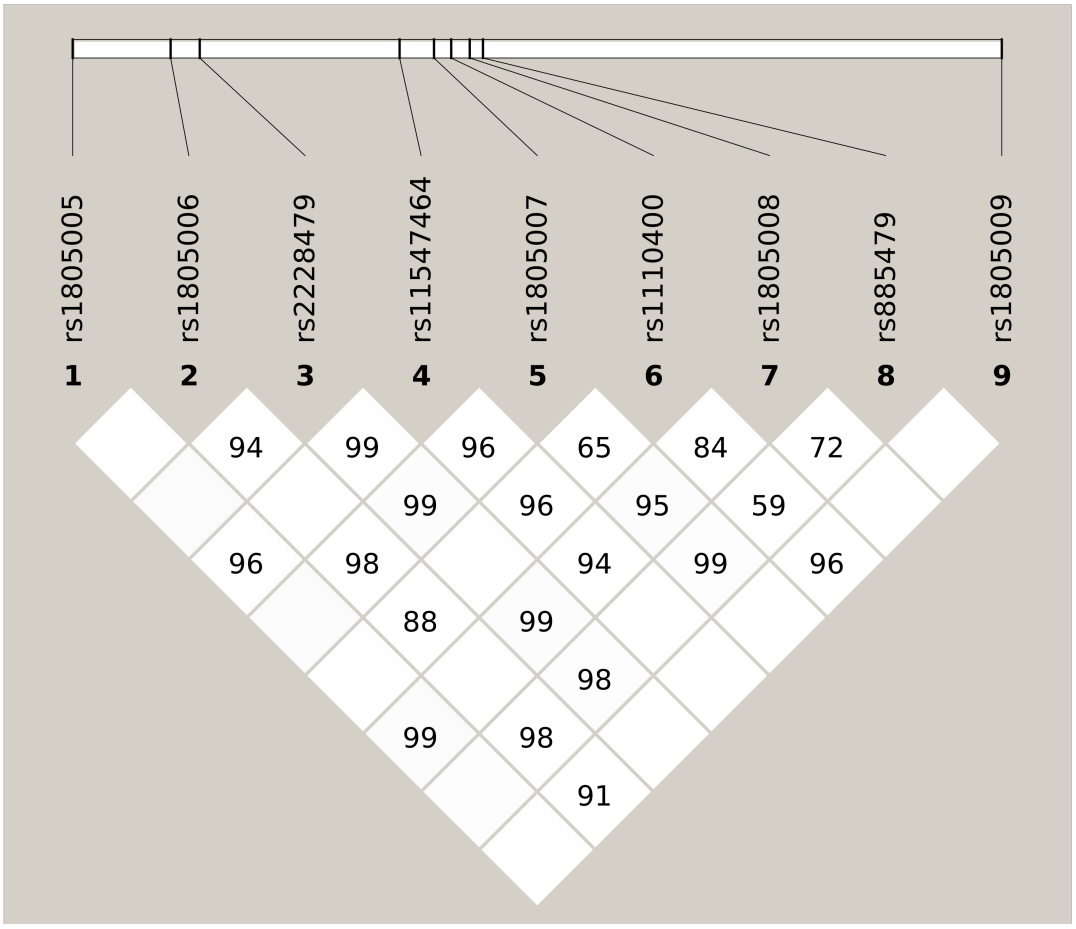


Figure 2.2: Linkage disequilibrium (LD) plot for twelve MC1R variants. Metric coding: value in diamonds = D'; Colour scheme: r-squared (white = 0; red = 1).

Haplotype	rs796296176	rs1805005	rs1805006	rs2228479	rs11547464	rs1805007	rs1805008	rs885479	rs1805009	rs555179612	rS201326893	rs1110400	HF
1	C	G	C	G	G	C	C	G	G	T	C	T	5.0e-1
2	C	**T**	C	G	G	C	C	G	G	T	C	T	1.2e-1
3	C	G	C	**A**	G	C	C	G	G	T	C	T	9.5e-2
4	C	G	C	G	G	**T**	C	G	G	T	C	T	8.9e-2
5	C	G	C	G	G	C	**T**	G	G	T	C	T	7.3e-2
6	C	G	C	G	G	C	C	**A**	G	T	C	T	4.9e-2
7	C	G	C	G	G	C	C	G	**C**	T	C	T	2.21e-2
8	C	G	**A**	G	G	C	C	G	G	T	C	T	1.25e-2
9	C	G	C	G	G	C	C	G	G	T	C	**C**	1.12e-2
10	C	G	C	G	**A**	C	C	G	G	T	C	T	7.0e-3
11	**CA**	G	C	G	G	C	C	G	G	T	C	T	4.2e-3
12	C	G	C	G	G	C	C	G	G	**TC**	C	T	1.6e-3

Table 2.3: Haplotypes of *MC1R* variants above MAF 0.01

In each haplotype the minor allele is highlighted in **boldface red**. The top haplotype is wild-type. Every other haplotype carries only one variant's minor allele.

Table 2.4: Haplotype associations for *MC1R* variants with red versus dark hair

Haplotype	Frequency	Effect (OR)	*P* value
1	5.0e-1	0.89	<2.0e-16
2	1.2e-1	3.36	<2.0e-16
3	9.5e-2	0.91	0.070
4	8.9e-2	143.74	<2.0e-16
5	7.3e-2	75.19	<2.0e-16
6	4.9e-2	0.92	0.256
7	2.21e-2	105.11	<2.0e-16
8	1.25e-2	56.26	<2.0e-16
9	1.12e-2	18.12	9.65e-9
10	8.7e-3	5.98	<2.0e-16
10	7.0e-3	82.43	<2.0e-16
11	4.2e-3	776.66	<2.0e-16
13	1.8e-3	1.20	<2.0e-16
12	1.6e-3	1004.25	<2.0e-16

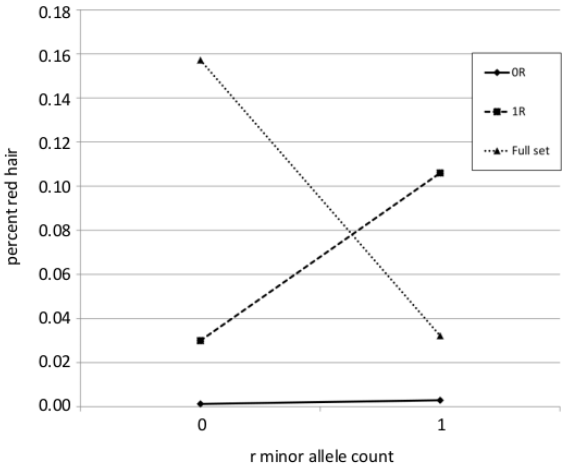
Effect size, here, odds ratio (OR) > 1 denotes a positive association with red hair. The most frequent haplotype (no.1 in Table 2.3) was used as the baseline against which all variant haplotypes were compared.

contribution of r alleles to red hair was truly negative or whether it was positive but dwarfed by that of R variants. To this end, we ran 2 types of analyses to test for the independent contribution of r variants to the red hair phenotype. First, we ran a haplotype association analysis and second, we ran regression on r allele count while holding the count of R minor alleles constant. Haplotype association analysis showed that each haplotype was positively associated with red hair (Table 2.4). In other words, each variant's minor allele, whether R or r, on a wild-type background of all other variants' major alleles in this haploblock was correlated with red hair. However, based on odds ratios, r variant contribution to red hair is up to 2 orders of magnitude lower than R variant contribution.

In the second set of analyses, we analysed for association of r variant minor alleles in the separate subsets of people with the total count of all R variant alleles equalling 0 and 1 and compared them to the full sample. The results, Figure 2.3, show that with ≤ 1 minor allele at all R variants considered together, r allele count is positively associated with red hair. In other words, r alleles mildly contribute to red hair on the background of a wildtype homozygous or a single heterozygous R genotype. In fact, the effect size of r variant count is higher on the background 1 R copy, suggesting that, expectedly, the contribution of r variants to red hair

Count of minor R alleles	Effect (OR)	p-value
0	2.2	2.7e-16
1	4.7	< 2.0e-16
Full set	0.18	< 2.0e-16

a



b

Figure 2.3: Interaction between r allele count and R allele count. Association for r allele count with red hair given an invariant R allele count in tabular (**a**) and graphical (**b**) format.

colour is stronger in individuals who already have 1 R allele. On the other hand, as single-variant analysis already shows (Table 2.2), in the whole sample r allele count is negatively associated with red hair.

To visualise this relationship, we plotted the red hair frequency distribution as a function of r allele count separately in two collapsed R allele count groups, 0 and 1, and in the full sample (Figure 2.3). We can see that r allele count is positively correlated with red hair in both 0R and 1R groups but negatively correlated with red hair in the full sample.

Our results demonstrate that while individually the two variant classes contribute to red hair, the r variant contribution is substantially milder than that of R variants by comparison of the magnitude of their effect coefficients. We posit that the correlation structure between R and r variants, namely r-squared close to 0 and D' close to 1, together with the high discrepancy in magnitude of effect, masks the true direction of association for the weaker-effect variants in single-variant analysis. The underlying direction of effect is revealed when all relevant variants are accounted for in haplotype analysis or, as we see below in Section 2.2.3, using multivariate regression analysis.

2.2.3 Best predictive *MC1R*-based model for red hair

Next, we sought to construct a model with the minimal number of *MC1R* variants in a GLM that would have the most predictive power for determining the expression of red hair. For comparison, we compiled a list of previous publications reporting

red hair prediction models (Table 2.5). We performed mRMR in a holdout set of 150,000 individuals to make an initial selection of variables, the top 10 of which were used in the most parsimonious GLMs (Table 2.6) and Supplementary Table 2.S1 for red versus dark). The AUROC values were 0.95 for red versus other and 0.97 for red versus dark. (To compare directly, we used the same 12 variants from 5 genes proposed in (35), which gave us an AUROC of 0.93 for red versus other and 0.96 for red versus dark.) Interestingly, while for the red versus dark comparison all *MC1R* variants have a positive association (OR $>$ 1) with red hair, Table 2.S1, in red versus other, the association for the two r variants (rs2228479 and rs885479) is negative (OR $<$ 1, Supplementary Table 2.6), signalling an effect flip when lighter hair colours – blonde and light brown – are grouped together with dark. We also performed LASSO regression on the complete set of imputed *MC1R* variants to take advantage of the innate attribute selection and coefficient penalisation of LASSO to minimise overfitting and maximise predictive performance. The LASSO models demonstrate the best performance we achieved in predictive modelling of the hair colour phenotype (Table 2.7 and Supplementary Table 2.S2 for red versus dark). The AUROC values for LASSO models were 0.96 for red versus other and 0.98 for red versus dark. In both non-LASSO GLMs the top 3 parameters are still the common R variants – rs1805007, rs1805008, and rs1805009 – followed by a combination of rarer R variants, 3 common r variants (rs2228479, rs885479, and rs1805005), and 2 frameshift R mutations (rs796296176 and rs555179612). In LASSO models we see all the known R and r variants as well as several more nonsynonymous variants and variants from 5' and 3' untranslated regions (UTRs).

Table 2.5: Previous models for red hair prediction.

Publication	Year	Phenotype	N	P	*MC1R* Vars	Other Vars	AUROC	Other Metric
Grimes et al.(18)	2001	red and auburn	197	0.274	[1] 4	0	n/a	[2] 0.960
Branicki et al.(36)	2007	red and blonde-red	184	0.410	[3] 2	0	n/a	[2] 0.975
Branicki et al.(37)	2007	red	390	0.240	[4] 3	0	n/a	[2] 0.960
Sulem et al.(38)	2007	red	[5] 6918	0.055	[3] 2	0	n/a	[6] 0.700
Walsh et al.(39)	2013	red, blonde-red,						
and auburn	1551	0.088	[7] 4	[8] 11	n/a	[9] 0.800		
Branicki et al. (40)	2011	red, blonde-red,						
and auburn	385	0.249	[10] 2	[11] 11	0.90	[12]		
Walsh et al. (41)	2014	red, blonde-red,						
and auburn	1601	0.085	[13] 11	[14] 4	0.92	n/a		
Sochtig et al. (35)	2015	red, blonde-red,						
auburn	605	0.14	[15] 5	[16] 7	0.94	[17]		
Caliebe et al. (42)	2016	red tint	400	0.31	[3]	n/a	0.75	[18]
Siewierska-Gorska								
et al. (43)	2017	red and blonde-red	186	0.24	[3]	[19]	0.84	[20]
Hysi et al. (44)	2018	red	[21] 15,015	[22]	[23] 8	[24] 268	[25] 0.87; [26] 0.84	[27] 0.35

N, sample size; P, red hair prevalence in the sample.

[1] rs796296176, rs555179612, rs1805006, rs11547464
[2] precision for variant homozygous or compound heterozygous redheads
[3] rs1805007, rs1805008
[4] rs1805007, rs1805008, rs11547464
[5] 5704 Icelanders and 1214 Dutch
[6] precision at 0.50 classification threshold

[7]rs201326893, rs796296176, rs1805006, rs11547464

[8]rs1042602 (TYR), rs4959270 (EXOC2), rs28777 (SLC45A2), rs683 (TYRP1), rs2402130 (SLC24A4), rs12821256 (KITLG), rs2378249 (ASIP), rs12913832 (HERC2), rs1800407 (OCA2), rs16891982 (SLC45A2), rs12203592 (IRF4)

[9]MLR highest probability hair colour category + a model for binary hair colour shade (light/dark) prediction; these two models used to make the final prediction, red-hair prediction accuracy, reported here.

[10]combined minor allele count (max. 2) at any R variant(rs201326893, rs796296176, rs1805006, rs11547464, rs1805007, rs1805008, rs1805009) or r variant (rs1805005,rs2228479,rs1110400,rs885479)

[11]rs12913832 (HERC2), rs12203592 (IRF4), rs1042602 (TYR), rs4959270 (EXOC2), rs28777 (SLC45A2), rs683 (TYRP1), rs1800407 (OCA2), rs2402130 (SLC24A4), rs12821256 (KITLG), rs16891982 (SLC45A2), rs2378249 (ASIP)

[12]sensitivity 0.78, specificity 0.95, precision 0.84, negative predictive value 0.93; 0.86 AUC for LASSO model.

[13]rs201326893, rs796296176, rs1805006, rs11547464, rs1805007, rs1805008, rs1805009, rs1805005, rs2228479, rs1110400, rs885479

[14]rs1042602 (TYR), rs4959270 (EXOC2), rs28777 (SLC45A2), rs683 (TYRP1)

[15]rs11547464, rs1805006, rs1805007, rs1805008, rs1805009

[16]rs28777 (SLC45A2), rs35264875 (TPCN2), rs1129038, rs12913832 (HERC2), rs4778138, rs7495174 (OCA2), rs12931267 (FANCA)

[17]Bayes classification

[18]sensitivity 0.19; specificity 0.09; accuracy 0.74; heritability for rs1805007 0.14 and for rs1805008 0.07

[19]rs16891982 (SLC45A2), rs12913832 (HERC2), rs1800401 (OCA2))

[20]sensitivity 0.67; specificity 0.93; accuracy 0.87; PPV 0.74; NPV 0.90

[21]7,291 QIMR (Brisbane Twin Nevus Study, Australian Twin Registry, and Tasmanian Eye Study) and 7,724 RS (Rotterdam Study)

[22]QIMR 0.054, RS 0.031, UKBB 0.047

[23]rs1805006, rs11547464, rs1805007, rs1805008, rs1805009, rs1805005, rs2228479, rs1110400

[24](39; 41) + 251 non-redundant variants in (44), Supplementary Table 9

[25]QIMR

[26]RS

[27]heritability

Table 2.6: Predictive multivariate GLM for red versus other hair colours

SNP ID	Function	MAF	Inf. score	Penetrance	Effect (OR)	*P* value
rs1805007	missense	1.03e-1	1.00	R	78.78	< 2.0e-16
rs1805008	missense	8.3e-2	1.00	R	35.32	< 2.0e-16
rs1805009	missense	2.78e-2	0.93	R	92.36	< 2.0e-16
rs2228479	missense	4.7e-3	1.00	r	0.80	2.6e-04
rs312262906	frameshift	9.7e-2	0.82	R	192.76	< 2.0e-16
rs11547464	missense	7.2e-3	1.00	R	46.93	< 2.0e-16
rs885479	missense	4.6e-2	1	r	0.78	6.1e-04
rs1805006	missense	1.22e-2	1.00	R	31.37	< 2.0e-16
rs555179612	frameshift	1.93e-3	1.00	R	225.45	< 2.0e-16
rs76337330	5'UTR	4.9e-3	0.98	ND	0.63	1.03e-10

The designations R, high-penetrance, and r, low-penetrance, are based on previously reported associations with red hair. Effect size, here, OR > 1 denotes a positive association with red hair, and OR < 1 denotes a negative association with red hair. Info score is a measure of imputation quality.

2.2.4 Use of *MC1R* genotypes to discriminate between non-red hair colours

Next, we constructed a series of models over pairwise dichotomous hair colour classes to determine whether *MC1R* genotype could predict other hair colours with appreciable power. GLMs were run starting with the top-ranked variant and successively adding other variants from the *MC1R* locus in decreasing order of mRMR score (data not shown). AUROC convergence plots are shown in Figure 2.4. While in all pairwise comparisons with red hair, models reached 0.90 AUROC with 10 or fewer variants, discrimination between other hair colours was poor, ranging from 0.55 to 0.68 AUROC.

2.2.5 Contribution of other genes to red hair

To test for the contribution of other previously reported hair pigmentation genes above and beyond *MC1R* variants, we took the top 10 mRMR-scored *MC1R* predictor variants and combined them with all of the variants from these other genes (Table 2.8), one gene at a time. mRMR was then run on that combined set. The resulting top 10 variants from *MC1R* and from each other gene were used to produce Figure 2.5a (red versus other) and Supplementary Figure 2.S1a (red versus dark). Figure 2.5b (red versus other) and Supplementary Figure 2.S1b (red versus dark) were generated by taking the top 10 variants from *MC1R* and the top 100 variants from the other genes and passing them to LASSO, which further performed its own inherent subspace selection. The subspace of variants from the other genes was restricted only to improve LASSO computational time, but because of the low

Table 2.7: MC1R variants selected by the LASSO model for red versus other hair colour

Variant	Function	MAF	Info score	RH association
rs1110400	missense	1.08e-02	0.98	Yes
rs11547464	missense	7.16e-03	1	Yes
rs148003355	5' UTR	2.72e-04	0.68	No
rs1805005	missense	1.11e-01	1	Yes
rs1805006	missense	1.22e-02	1	Yes
rs1805007	missense	1.03e-01	1	Yes
rs1805008	missense	8.25e-02	1	Yes
rs1805009	missense	2.78e-02	0.93	Yes
rs199920775	synonymous	1.76e-04	0.66	No
rs200000734	missense	5.44e-04	1	Yes
rs200050206	missense	6.50e-04	0.55	No
rs201326893	frameshift	2.56e-04	1	Yes
rs202197434	frameshift	1.47e-04	0.45	No
rs2228478	nonsynonymous	1.15e-01	0.996	No
rs2228479	missense	9.72e-02	1	Yes
rs796296176	frameshift	5.51e-03	0.82	Yes
rs3212359	5' UTR	3.15e-01	0.99	No
rs3212361	5' UTR	2.43e-01	0.99	No
rs3212371	5' UTR	1.14e-01	0.99	No
rs3212379	5' UTR	7.46e-03	0.87	No
rs34158934	missense	2.61e-04	0.63	No
rs34474212	missense	4.80e-05	0.92	No
rs34490506	synonymous	1.89e-04	0.53	No
rs367985661	synonymous	8.00e-06	0.09	No
rs368507952	missense	4.83e-04	1	Yes
rs374423188	missense	4.53e-05	0.42	No
rs376670171	missense	4.00e-05	0.31	No
rs555179612	frameshift	1.93e-03	1	Yes
rs572754025	3' UTR	3.47e-05	0.41	No
rs577907985	5' UTR	7.54e-04	0.53	No
rs765283788	3' UTR	2.88e-04	0.86	No
rs868197501	5' UTR	4.00e-05	0.75	No
rs885479	missense	4.62e-02	1	Yes

Effect size, here, odds ratio (OR) > 1 denotes a positive association with red hair. Info score is a measure of imputation quality, and the "RH association" column shows whether a red hair association had been previously published.

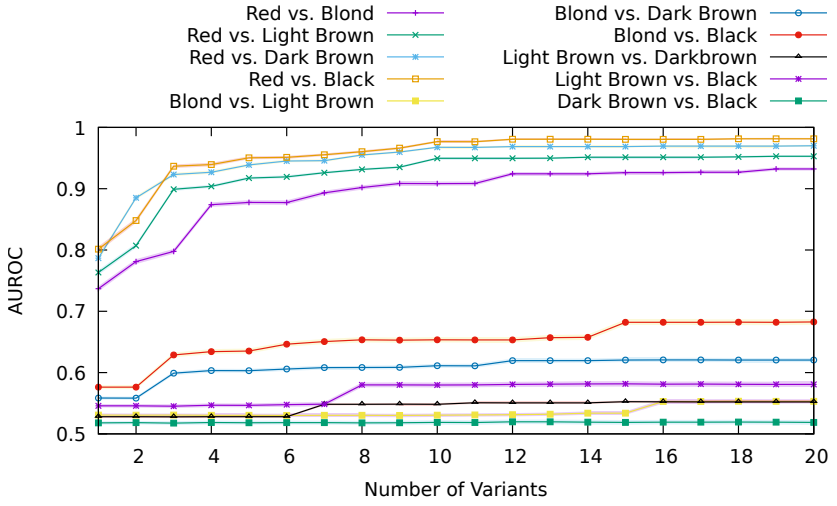
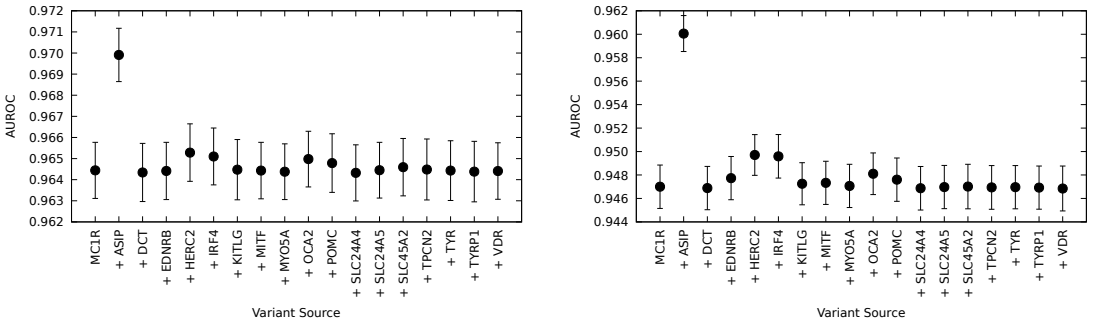


Figure 2.4: Area under the receiver operating characteristic (AUROC) curves for all pairwise hair colour comparison GLMs (generalised linear models) with *MC1R* variants as predictors.

information of the remaining variants, excluding them from LASSO had no effect on the final models. In all cases, attribute selection was preformed strictly without knowledge of the testing set.

Figure 2.6 and Supplementary Figure 2.S2 show the relative importance of other genes over and above *MC1R* in determining red hair. Based on the statistically highly powered paired-sample t-test, we can confidently reject the null hypothesis that model performance with variants from other genes is the same as *MC1R*. In order to determine whether this difference is meaningful in terms of real predictive capacity, we compared the performance of our LASSO models to models using top 10 *MC1R* variants plus top 100 variants from 1000 randomly selected genes to measure predictive performance in red versus other hair colours (Figure 2.6) and red versus dark hair colour (Supplementary Figure 2.S2). The same subset of genes composes the data for both figures. It was constructed by taking the complete list of identified genes from the NCBI database and performing a completely unbiased, pseudorandom selection of 1000 members. Several of the genes with a previously reported role in hair colour perform worse than the *MC1R*-only model, and their statistically significant deficiency is due to overfitting irrelevant noise in the training sets within the cross-validation procedure. Several that perform better lie within the 2σ confidence interval for the distribution based on random genes. In red versus other hair colour prediction, *ASIP*, *HERC2*, *OCA2*, and *IRF4*, and to a lesser extent *POMC*, *SLC45A2*, (and in red versus dark hair colour, *ASIP*, *HERC2*, *OCA2*, and to a lesser extent *POMC*, *SLC45A2*, and *TYR*) provide a lift to the models' AUROC



(a) GLM (b) LASSO

Figure 2.5: Area under the Receiver Operating Characteristic (AUROC) curves for (a) mRMR and (b) LASSO models using 10 top MC1R genetic variants and 10 top genetic variants from each gene for mRMR and 100 top genetic variants from each gene for LASSO. Red versus other hair colour. For both mRMR and LASSO, the model performance for all genes is statistically significantly different from the model using only *MC1R* variants. Despite the 10 iterations of 10-fold cross-validation to obtain an estimate of mean ROC performance, error bars for the 95 % confidence interval are based on a standard error of the mean assuming a sample size of 10 rather than 100 due to lack of test set independence in folds between cross-validation iterations.

Table 2.8: Previously reported hair colour genes.

Gene	Gene name	Citation
MC1R alone	Melanocortin-1 receptor	*NA*
ASIP	Agouti signaling protein	(39; 45–48)
DCT	Dopachrome tautomerase	(49; 50)
EDNRB	Endothelin receptor type B	(51)
HERC2	HECT and RLD domain containing E3 ubiquitin protein ligase 2	(40; 42; 52–54)
IRF4	Interferon regulator factor 4	(39; 40; 42; 55)
KITLG	KIT ligand	(39; 40; 49; 55)
MYO5A	Myosin VA	(49; 50)
OCA2	OCA2 melanosomal transmembrane protein	(40; 43; 52–54)
SLC24A4	Solute carrier family 24 member 4	(34; 38–40; 42; 48; 55)
SLC24A5	Solute carrier family 24 member 5	(50)
SLC45A2	Solute carrier family 45 member 2	(35; 39; 40; 48–50; 56; 57)
TPCN2	Two pore segment channel 2	(35; 47; 55)
TYR	Tyrosinase	(10; 39; 40; 48; 49; 52; 52; 58; 59)
TYRP1	Tyrosinase-related protein 1	(40; 60; 61)

that lies outside the 2σ confidence interval. Even the best of the models including variants from another gene, *MC1R* with *ASIP*, is only a 0.57 % improvement in AUROC for red hair versus other hair colour and 0.44 % improvement in AUROC for red hair versus dark hair colour, which we deemed insufficient to sacrifice parsimony. In short, almost the entirety of the variation in the disease-free red hair phenotype is explained by *MC1R* variants alone; only 10 *MC1R* variants are sufficient to obtain the best predictive capacity yet reported, and 30 *MC1R* variants in a LASSO model can perform even better.

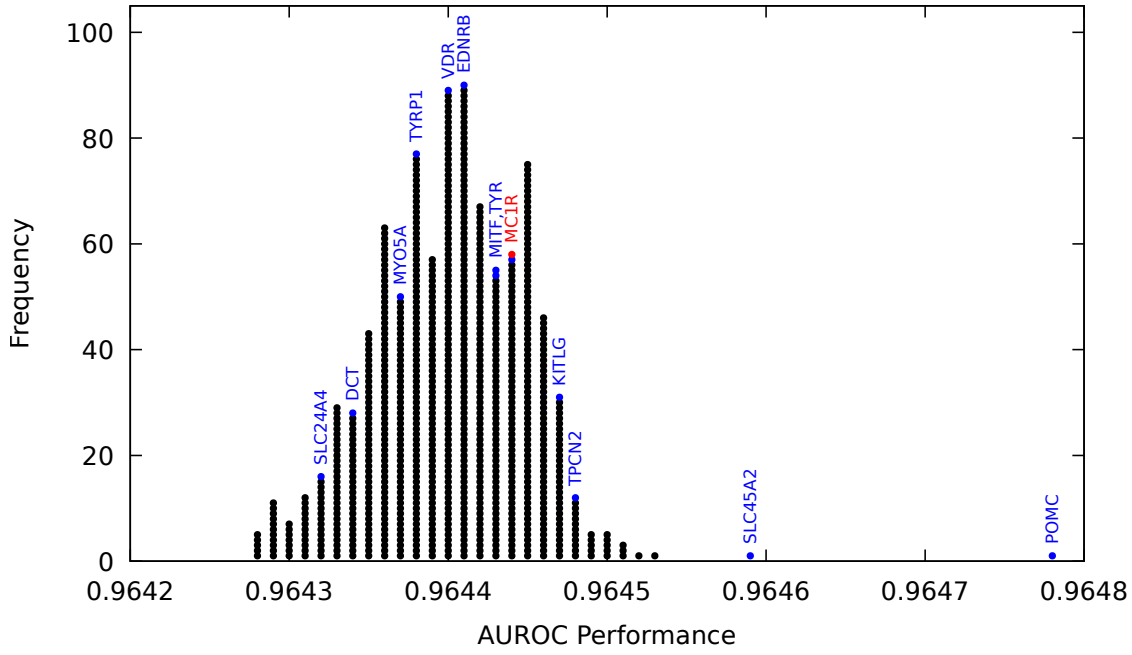


Figure 2.6: Red versus other hair colour prediction using LASSO models with 10 *MC1R* and 100 top mRMR-ranked variants from 1000 randomly selected genes. All the genes shown fall within 2σ. *ASIP*, *OCA2*, *IRF4*, and *HERC2* (not shown), have AUROC values 0.970, 0.965, 0.965 and 0.965, respectively, and are the only genes whose variants improve predictive performance above and beyond MC1R variants. The variants of these four genes and two other genes outperform *MC1R*-alone models with a statistically significant difference (t-test *P*-values: *ASIP*, <1e-16; HERC2, <1e-16; *OCA2*, <1e-16; *IRF4*, <1e-16; *POMC*, 5.7e-10; *SLC45A2*, 8.3e-3.

2.3 Discussion

Here we present an in-depth analysis of the relationship between *MC1R* variants and the red hair phenotype. Testing common and rare variants across the entire gene locus has confirmed previous reports of nonsynonymous missense variants as the primary effectors of the red hair phenotype with additional contribution from frameshift mutations. However, although all prior studies agreed on the contribution of R alleles to red hair colour, the contribution of r variants has seen conflicting evidence. Most reports have shown either a weak association with red hair or no impact on hair colour for r alleles. Additionally, association between r alleles and dark hair (39; 62) and darker skin (63) has been documented. Lastly, a negative association between r alleles and red hair also has precedents. One group reported that a comparison between r allele carriers (rs1805005 and rs2228479) and R allele carriers/wildtype group showed a correlation with lower red colour component in hair for the former (49), and two groups reported an $OR < 1$ for r with red hair: first Raimondi et al. in 2008 (64) and most recently, during the preparation of this manuscript, Morgan *et al.*, who also noted that this OR changed if R variants were included in the regression model (65).

Addressing conflicting previous reports regarding R and r variants, we determined that, when isolated in an invariant R minor allele count subsample, r variant alleles do contribute to red hair, although their contribution is much weaker by comparison to R variants. Haplotype analysis demonstrated that no two variant alleles among all R and r variants effectively co-occur; therefore, because a higher count of r alleles lowers the chance of R allele presence in a particular individual, regression on just r allele count results in a negative association with red hair. These findings are summarised in Figure 2.3. Particularly, in our study, the dichotomous dependent variable "red versus dark hair" resulted in a negative association for r alleles with red hair and by default positive with dark hair Table 2.2.

An important drawback of variant-centered analysis is that in the presence of strong haplotypic effects, as in the *MC1R* case, the marginal variant associations are not generalisable to other populations where haplotype frequencies are different, because the effect size or even direction are influenced by those frequencies. However, from the epidemiological perspective, a carrier of a variant that is marginally protective relative to the rest of the sample population under study has a smaller risk of disease than a random person from the same population. This effect is real and if strong enough can be practically predictive of reduced risk of disease. At the same time, the same variant allele can be deleterious in comparison to the allele on the background of ancestral haplotypes.

The high LD between R and r variants, together with the high discrepancy in magnitude of effect, contribute to the observed effect reversal when diplotypes of the entire MC1R locus are reduced to essentially single variants with one of the alleles being r. This effect reversal is known as the flip-flop phenomenon (66; 67). A flip-flop may take place whenever there is a joint effect of multiple variants acting on a phenotype but only a subset of them is scored and analysed for single-variant genetic association with the phenotype. Both large variation in effect size among haplotypes and high LD facilitate the effect reversal, but neither is required for it to occur. From a population genetics point of view, a single-variant effect size for the variant's minor allele is analogous to the marginal effect. It is the weighted average of the effect sizes of all diplotypes that carry this minor allele, with the weights given by the population frequencies of those diplotypes (68). Under the Hardy-Weinberg equilibrium, the weights are the population frequencies of haplotypes, and they are among key factors that trigger the effect reversal, together with LD and variability in effect size among haplotypes. Therefore, an important outcome of our study is the discovery that the small-effect variants of the MC1R locus have their direction of effect flipped in single-variant association. Alternatively, when these r variants are used as predictors for red versus dark hair in a multivariate GLM that includes all relevant variants, the direction of effect for all of them bespeaks a positive correlation between their minor alleles and red hair. This study thus exemplifies a phenomenon emergent in a large population with many rare genetic variants of strong effect size, in which the population phenotypic mean (for example, prevalence of disease) may become sufficiently elevated for the weaker rare-susceptibility variants to appear protective on the background of the overall prevalence of disease. Caution is always advisable in interpreting the direction of effect in genome-wide association analyses without considering that joint effects of many loci may be at work.

We also tested *MC1R* variants in pairwise comparisons for all available hair colour phenotypes to determine their possible contribution to colours other than red. In addition to the associations with darker hair and skin for rs1805005 and rs2228479 mentioned above, prior publications have reported weak association for rs1805005 with blonde hair in (22; 36; 59). However, our results show that compared to red hair, the predictive power of *MC1R* variants for other hair colours is very weak (Figure 2.4) and could not be reliably used for the purposes of identifying a missing individual's phenotype. The higher AUROC for light-dark hair colour models compared to light-light and dark-dark could be explained by some overlap between strawberry blonde and blonde as well as auburn with light brown, thereby giving some discriminatory power to the model for the red component in the latter

hair colour in each pair. *MC1R* variant alleles are, expectedly, least informative in discriminating between dark brown and black hair, neither of which is likely to be contaminated by red hair colour.

Since 2001, *MC1R* variants have been exploited in forensic science to predict hair colour of missing individuals in police investigations. Of the relevant publications summarised in Table 2.5, the first two (18; 36) relied on exclusively *MC1R* variants and contingency tables for red hair colour prediction. While their precision of 96 and 97.5 %, respectively, for R homozygous or compound heterozygous genotypes predicting red hair is high, it is notable that their sample was enriched for redhaired individuals (27 % and 41 %, respectively) and not representative of the general population (2-5 %). Thereafter, focus shifted from predicting only red hair to determining hair colour, and other genes were included (35; 39–41; 43; 44). Among these reports, ones that used a more representative proportion of red-haired individuals (35; 38; 39; 41; 44) and AUROC as the performance metric (40–44), the best-performing model gave an AUROC of 0.94 for red versus other hair colour (35).

Harnessing the high-powered UKBB sample available to us we attempted to improve the predictive model for discriminating between red and non-red hair colour using only *MC1R* variants. While all previously published predictive models included variants from other genes and were nevertheless only able to obtain an AUROC of 0.94 for the red versus all other at best (35), our parsimonious GLM, which took only 10 *MC1R* variants as predictors, yielded an AUROC of 0.95 for red versus all other hair colours (Figure 2.5a), and an AUROC of 0.97 for the most distinct class comparison, red versus dark (Supplementary Figure 2.S1a). Our less parsimonious but still only *MC1R*-based LASSO model yielded an AUROC of 0.96 for red versus other (Figure 2.5b) and 0.98 for red versus dark (Supplementary Figure 2.S1b). Thus, our results show that it is possible to construct a model with near-perfect predictive capacity on *MC1R* variants alone. In confirmation of many previous findings, the top three predictors in the parsimonious GLM are R variants rs1805007, rs1805008, and rs1805009. While two r variants rs2228479 and rs885479 are included in this model, rs1805005 is notably absent. The remaining predictors are rarer R variants or frameshift mutations.

Notwithstanding the unprecedented AUROC values of 0.95 and 0.96 obtainable from *MC1R* variants in the red hair colour prediction using GLM and LASSO, respectively, we also checked whether adding variants from other genes might improve discrimination between red and dark hair. The addition of mRMR-ranked top variants from *ASIP*, *HERC2*, *OCA2*, and *IRF4* did provide additional predictive capacity, while the addition of variants from other candidate genes was no better

than randomly selected genes. The additional predictive capacity, although statistically significant, represented a mere 0.57 % increase in AUROC in the best case (*ASIP*), which we do not interpret as phenotypically meaningful. A recent hair colour GWAS, also done on the UKBB, by Morgan *et al.* stipulates that including variants from 8 other loci throughout the genome improves by 17 % the heritability estimate obtained using only *MC1R* variants (65). These estimates use narrow-sense heritability (h2) and are therefore only sensitive to additive effects, which account for a fraction of the explanatory power of recessive and negatively-linked *MC1R* variants.

A limitation of our study is that the phenotype of interest, hair colour, was obtained by self-report and its identification could be refined by more objective, quantitative methods. However, by relying on subjective human determination of hair colour, we approximate a real-life situation in which this information would be based on observation rather than an objective pigment quantification method.

In conclusion, our findings may be summarised in five parts. First, we have identified an effect reversal in conventional single-variant analysis when in fact both weak- and strong-effect variants contribute toward the red hair phenotype. We predict that this reversal is more frequent in loci with a single-variant-per-haplotype architecture, similar to *MC1R*, especially given considerable discrepancy in effect sizes between haplotypes. Second, we have confirmed a positive independent association for each of the previously reported nonsynonymous *MC1R* variants with red hair. Third, we offer for the purposes of red hair colour identification, for example in a forensic setting, a robust, parsimonious and therefore economical predictive GLM with an unprecedented performance metric of AUROC 0.95 for which only 10 *MC1R* variant loci are needed. An even better performance metric of 0.96 is obtainable by still only using LASSO-derived genetic variants within the short *MC1R* locus. Fourth, we have shown that *MC1R* does not contribute significantly to hair colours other than red. Lastly, we conclude that contribution from other hair-colour-related genes to red hair colour is negligible and posit *MC1R* as the sole substantial genetic contributor to red hair colour.

2.4 Materials and Methods

2.4.1 UKBB cohort

Our study cohort comes from the U.K. Biobank (UKBB), a repository of genotypes and phenotypes from 500,000 participants aged 40-69, recruited between 2006 and 2010 (application 20802). Genotyping was done on one of two 95 %-overlapping arrays – Affymetrix UK BiLEVE Axiom and Affymetrix UK Biobank Axiom – containing 820,000 single-nucleotide variants. For all analyses we used the imputed genotypes for Caucasian individuals, as specified in the UKBB Data Field, hereafter DF, 22006. Quality control filters for heterozygosity rate (DF22010) as well as sex mismatch, variant call rate, unintended duplicates, and outliers of >10 standard deviations in ancestry principal component analysis, PCA (DF22051) were applied, and individuals who withdrew from the study were removed, yielding an effective number of 402,000 participants. Hair colour (DF1747) was provided by self-report. Participants were asked to select one of five choices to describe their natural hair colour before greying: blonde, red, light brown, dark brown, or black.

2.4.2 Statistical analyses

Model parameters: genotypes, phenotypes, and covariates

For the analyses described below, we used all available variants (post-imputation) for each gene, and gene locus boundaries were defined according to chromosomal boundaries provided in the Gene Database hosted by the U.S. National Center for Biotechnology Information (NCBI), (69), Genome Reference Consortium Human genome build 37 (GRCh37). Genotypes (one or more genetic variant minor allele counts) were used as independent variables, and the phenotype (hair colour) as the dependent variable. Covariates were used as described below, and regression coefficients were transformed into odds ratios.

Association analysis: GLM

We used two different modelling paradigms consistent with distinct goals. Given that the first goal was to demonstrate the statistical significance and effect magnitude and direction of associations, we applied generalised linear models (GLMs). Without a model evaluation step there was no need for a testing set; therefore, we used the entire cohort (500,000). Additionally, we used covariates (age, sex, recruitment site, and 40 ancestry principal component (PC) vectors) to account for population stratification and dichotomised the hair colour phenotype, “red versus dark”, where the “dark” category comprised dark brown and black hair colours. Given that the

goal for this association analysis was to isolate the effect of genetic variants on red hair, blonde and light brown were withheld to maximise phenotype homogeneity, thereby avoiding possible overlap between red and strawberry blonde or auburn brown hair colour, which have likewise been reported to be mediated by *MC1R* variants.

Predictive capacity assessment: mRMR and LASSO

For the second goal, which was to demonstrate the predictive capacity of our models, we ran all possible pairwise hair colour comparisons, as well as "red versus other". For these analyses we did not use covariates, given the intention to determine how well a restricted set of genetic variants alone could discriminate between possible hair colours or determine the donor's hair colour to be red, with no other information provided, as may be the case in a forensic investigation. We performed attribute selection using two different methods: the minimum redundancy maximum relevance (mRMR) algorithm (70) and the least absolute shrinkage and selection operator (LASSO) (71). mRMR is valuable as an attribute ranking algorithm, ordering potential predictors by mutual information with the class variable penalised by average mutual information with previously selected attributes. LASSO performs variable selection and regularisation to combat over-fitting and produce parsimonious models with the aim to select an optimal covariate subspace. We used mRMR to demonstrate performance convergence of the prediction task with an increasing number of variants from *MC1R* and to filter the space of candidate variants to use in LASSO to decrease computational workload.

Training and testing sets

To avoid information leakage between attribute selection and subsequent model construction, we divided our data set consistently with the UKBB genotype release schedule. The initial release (2016) – data for 150,000 individuals – was used as a holdout set for mRMR analysis, and the model was constructed and evaluated using the remaining 350,000 individuals from the full release (2017). Specifically, we split the remaining 350,000 individuals into 10 different sets of 10 mutually exclusive folds and alternately used every agglomeration of 9 folds in each set to predict hair colour in the remaining fold. LASSO feature selection was done exclusively within its training data. For each class under consideration, cross-validation folds were invariant across models, so the repeated cross-validation provided 100 paired samples of any given performance metric. Though these samples were not strictly independent, they can be used for confidence interval construction and model comparison tests. The confidence intervals in Figure 2.5 and Supplementary Figure 2.S1

were thus derived, and comparisons between models using only *MC1R* variants and models also incorporating variants from other genes are based on a dependent t-test for paired samples across these 100 prediction subsets.

Model performance metric

In contrast to the studies that report threshold-dependent metrics, such as accuracy or precision, we use the area under the receiver operating characteristic curve (AUROC) as a model performance metric for the following reasons. First, the class balance ratio changes across predictive tasks depending on the implicated hair colours. The AUROC metric is invariant to the class balance ratio and can therefore be used as a common interpretable performance characteristic of models to produce informative visualisations, such as Figure 2.4, that meaningfully demonstrate the relative difficulty of the predictive task irrespective of class imbalance. Second, commonly used measures such as accuracy can be misleading, since trivial predictors can report near-optimal performance on highly imbalanced classes by always predicting the majority class. Hair colour class balance ratios can approach 50:1 and are therefore subject to apparent distortion in the accuracy metric when reporting binary class predictive performance. The third and final reason is that measures such as accuracy, precision, and recall are derived from a single instantiation of the classification confusion matrix, and there are as many such legitimate instantiations as discrete probabilities in the predictive output. While it is typical to use the confusion matrix resulting from a $P(+) > 0.5$ probability threshold, we offer instead AUROC as a measure of performance that does not require us to make a potentially suboptimal choice in trade-offs such as sensitivity versus specificity, a choice best left in the hands of potential users of this information for practical purposes, such as in forensic investigations.

Software

Software to perform these analyses included R version 3.4.4 with the "caret" (72) and "glmnet" packages (73). mRMR analyses were conducted with validated software written by R.N.L. to improve the mRMR (70; 74) reference implementation and Fast-mRMR (75) for efficient and flexible usage on the UKBB data set. This software is publicly available on GitHub [`https://github.com/rlichtenwalter/mRMR`].

Haplotype association analysis was performed using the haplo.stats R (76) package. Linkage disequilibrium (LD) was visualised using the Haploview software (77).

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2.6 Supplementary Material

Table 2.S1: Predictive multivariate GLM for red versus dark hair

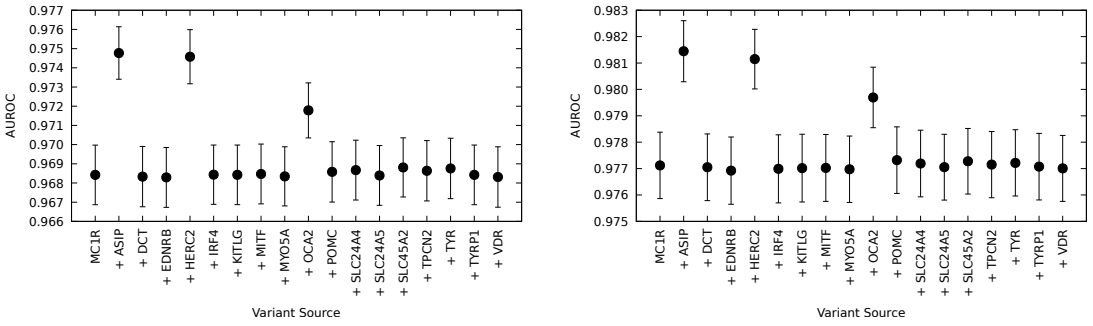
SNP ID	Function	MAF	Info score	Penetrance	Effect (OR)	P value
rs1805007	missense	1.03e-1	1	R	248.56	< 2.0e-16
rs1805008	missense	8.5e-2	1	R	119.35	< 2.0e-16
rs1805009	missense	2.78e-2	0.93	R	230.44	< 2.0e-16
rs2228479	missense	9.7e-2	1	r	1.36	1.68e-06
rs796296176	frameshift	5.5e-3	0.82	R	537.33	< 2.0e-16
rs11547464	missense	7.2e-3	1	R	123.77	< 2.0e-16
rs885479	missense	4.6e-2	1	r	1.28	0.00443
rs1805006	missense	1.22e-2	1	R	91.83	< 2.0e-16
rs555179612	frameshift	1.93e-3	1	R	720.15	< 2.0e-16
rs1805005	missense	1.11e-1	1	r	5.33	< 2.0e-16

The "Penetrance" designations R=strong and r=weak are based on previously reported associations with red hair. Effect size, here, odds ratio (OR) > 1 denotes a positive association with red hair, and OR < 1 denotes a negative association with red hair. Info score is a measure of imputation quality.

Table 2.S2: MC1R variants selected by the LASSO model for red versus dark hair colour

Variant	Function	MAF	Info score	RH association
rs530536156	5' UTR	1.83e-03	0.78	No
rs3212379	5' UTR	7.46e-03	0.87	No
rs3212359	5' UTR	3.15e-01	0.99	No
rs3212361	5' UTR	2.43e-01	0.99	No
rs577907985	5' UTR	7.54e-04	0.53	No
rs796296176	frameshift	5.51e-03	0.82	Yes
rs200050206	missense	6.50e-04	0.55	No
rs1805005	missense	1.11e-01	1	Yes
rs34474212	missense	4.80e-05	0.92	No
rs1805006	missense	1.22e-02	1	Yes
rs2228479	missense	9.72e-02	1	Yes
rs34158934	missense	2.61e-04	0.63	No
rs11547464	missense	7.16e-03	1	Yes
rs374423188	missense	4.53e-05	0.42	No
rs1805007	missense	1.03e-01	1	Yes
rs201326893	missense	2.56e-04	1	Yes
rs1110400	missense	1.08e-02	0.98	Yes
rs1805008	missense	8.25e-02	1	Yes
rs885479	missense	4.62e-02	1	Yes
rs376670171	missense	4.00e-05	0.31	No
rs555179612	frameshift	1.93e-03	1	Yes
rs199920775	synonymous	1.76e-04	0.66	No
rs200000734	missense	5.44e-04	1	Yes
rs34490506	synonymous	1.89e-04	0.53	No
rs202197434	missense	1.47e-04	0.45	No
rs1805009	missense	2.78e-02	0.93	Yes
rs368507952	missense	4.83e-04	1	Yes
rs2228478	synonymous	1.15e-01	0.996	No
rs572754025	3' UTR	3.47e-05	0.41	No
rs765283788	3' UTR	2.88e-04	0.86	No

Effect size, here, odds ratio (OR) > 1 denotes a positive association with red hair. Info score is a measure of imputation quality, and the "RH association" column shows whether a red hair association had been previously published.



(a) GLM (b) LASSO

Figure 2.S1: Area under the receiver operating characteristic (AUROC) curves for (a) mRMR and (b) LASSO models using 10 top *MC1R* genetic variants and 10 top genetic variants from each gene for mRMR and 100 top genetic variants from each gene for LASSO. Red versus dark hair colour. For both mRMR and LASSO, the model performance for all genes is statistically significantly different from the model using only *MC1R* variants. Despite the 10 iterations of 10-fold cross-validation to obtain an estimate of mean ROC performance, error bars for the 95 % confidence interval are based on a standard error of the mean assuming a sample size of 10 rather than 100 due to lack of test set independence in folds between cross-validation iterations.

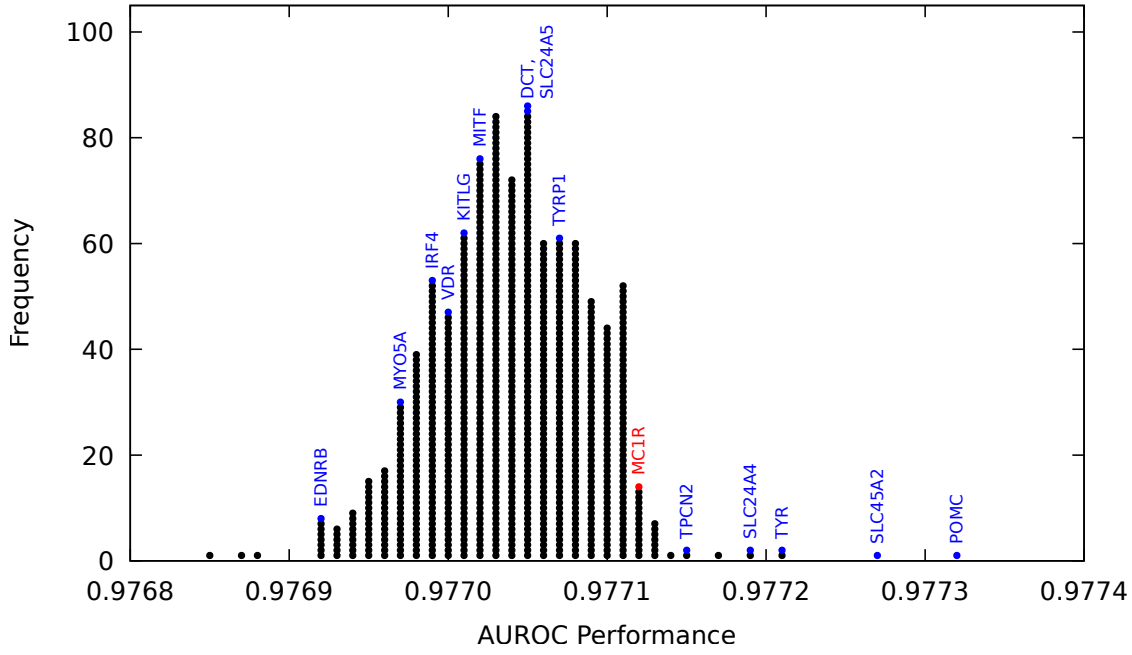


Figure 2.S2: Red versus dark hair colour prediction using LASSO models with 10 *MC1R* and 100 top mRMR-ranked variants from 1000 randomly selected genes. All the genes shown fall within 2σ. *ASIP*, *HERC2*, and *OCA2* (not shown), have AUROC values 0.981, 0.981, and 0.980, respectively, fall outside of 2σ. The variants of these three genes and three other genes outperform *MC1R*-alone models with a statistically significant difference (t-test P values: *ASIP*, <1e-16; HERC2, <1e-16; *OCA2*, <1e-16; *POMC*, 3.3e-5; *SLC45A2*, 1.1e-3; *TYR*, 3.3e-2).

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Linking statement to Cherapter 3

While Chapter 2 elucidates the relationship between *MC1R* variants and red hair colour, the manuscript included as Chapter 3 tests the association of *MC1R* variants with pain sensitivity, thereby completing the relationship of association between *MC1R* genotypes, red hair colour, and pain sensitivity. Although our original intention was to use a statistically derived genotypic construct from the red hair study to elucidate the effects of this gene on pain sensitivity, the difference in primary responsible regions within *MC1R* for these two phenotypes precluded this possibility. Instead we learned that while red hair is primarily mediated by nonsynonymous variants, pain sensitivity appears to be affected by the variants in the regulatory 5'-UTR. Interestingly, the discovered effects suggest that although both phenotypes are statistically associated with this genetic locus, the different regions and their main functions – defining protein composition versus affecting transcript levels – may be contributing to the pathways directly responsible for these phenotypes.

3

Disentangling the contribution of MC1R to pain and red hair.

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Genetic variation in melanocortin-1 receptor (MC1R) has a known role in red hair. Studies on responses to noxious stimuli in red-haired individuals have also been conducted, but the findings are mixed. To investigate a possible divergence between variants responsible for red hair and pain sensitivity, we performed a gene-wide association analysis in the Orofacial Pain: Prospective Evaluation and Risk Assessment (OPPERA) cohort. All genotyped *MC1R* variants were tested for association with heat pain temporal summation and sensitivity. Our analyses showed an association for pain sensitivity with the 5'-UTR, tagged by rs3212361, and one nonsynonymous variant, rs885479 (R163Q), previously shown to be weakly associated with red hair. For both variants, the minor allele was protective. These results were validated in the 500,000-person U.K. Biobank (UKBB) cohort, where the minor alleles of rs3212361 and rs885479 were associated with a reduced count of persistent

pain conditions as well as individual pain conditions. Haplotype association analysis revealed a possible joint effect from the two individual variants. The 5'-UTR variant rs3212361 was further identified as an expression quantitative trait locus (eQTL), associated with reduced transcript levels of *MC1R* in the brain and in the peripheral tibial nerve. Hair colour association analysis of the loss-of-function rs3212361 allele identified the expected association with red hair, and red hair colour itself was associated with a reduced count of persistent pain conditions. Together, our results suggest that different mechanisms mediated by genetic variation in the *MC1R* locus contribute to red hair and pain.

3.1 Introduction

The gene *MC1R* encodes the melanocortin-1 receptor, MC1R, a G protein-coupled receptor (GPCR) responsible for skin and hair pigment biosynthesis (1). Once endogenously activated by the melanocyte stimulating hormone, α-MSH, or the adrenocorticotropic hormone, ACTH, MC1R signals via cAMP upregulation for a switch from the default pheomelanin to eumelanin production (2; 3). The ratio of eumelanin to phaeomelanin determines hair colour, and a functionally compromised MC1R leads to red hair colour (4).

While its connection to red hair has been known for decades, *MC1R*'s involvement in pain was first reported in the early 2000s, by Mogil and colleagues (5; 6). They showed a favourable response to opioid analgesics in individuals with at least two *MC1R* variants – previously reported as associated with red hair – undergoing experimental heat, ischemic, and electrical current sensitivity protocols. The later publication also showed a lower baseline sensitivity to electrical current pain in the two *MC1R*-variant allele group. By contrast, two contemporary studies by Liem and colleagues found an increased anaesthetic requirement in red-haired individuals (7; 8), with a higher baseline sensitivity to hot and cold stimuli (8).

Given that red hair is known to result from loss-of-function (LOF) variants, we set out to test three possible explanations for these apparently divergent findings: 1. LOF in *MC1R* leads to reduced sensitivity to pain (in agreement with Mogil's publications); 2. LOF in *MC1R* leads to increased sensitivity to pain (in agreement with Liem's publications); 3. red hair and pain sensitivity are mediated by different but correlated variants in *MC1R*.

The studies done by Mogil and Liem suggest that *MC1R* may affect the response to a variety of noxious stimuli. Therefore, it is plausible that MC1R's polymorphisms mediate nociception at the point of noxious stimulus integration – the spinal dorsal horn – or higher in the ascending or descending pain signalling pathway. To capture this activity at the first hub of pain processing, we selected two measures from the heat pain wind-up paradigm: temporal summation and pain sensitivity. The heat protocol was chosen as the only shared modality affected by MC1R reported in Mogil's and Liem's studies.

To address the possibility that different yet correlated genetic variants were responsible for hair colour and pain sensitivity, we included all common genotyped MC1R variants in the discovery cohort analysis (9). For validation, we used the U.K. Biobank (UKBB) dataset. While no QSTs were tested in this cohort, a surrogate pain phenotype was available – a count of persistent clinical pain conditions. The mechanisms underlying nociceptive temporal summation and pain sensitivity have been shown to overlap with the mechanism responsible for central sensitisation (10; 11), the facilitation of both of which increases one's risk for developing chronic pain (12; 13). The availability of hair colour data in this cohort was an additional benefit and allowed us to query directly the relationship between these two phenotypes as mediated by genotype.

3.2 Materials and Methods

3.2.1 Cohorts

The association analysis was first conducted in the Orofacial Pain: Prospective Evaluation and Risk Assessment (OPPERA) cohort (9). In this prospective study of the temporomandibular disorder (TMD), participants aged 18-44, recruited from 4 U.S. study sites – the University of Maryland at Baltimore, the University of Buffalo (New York), the University of North Carolina at Chapel Hill, and the University of Florida at Gainesville – were enrolled from 2006 to 2008 and diagnosed for the presence of TMD at recruitment and follow-up. Additionally, participants were characterised for a battery of quantitative sensory testing (QST) measures to determine sensitivity to heat, mechanical cutaneous, and pressure pain. Genotyping was done at 2.5 million single variant positions on the Illumina Infinium Omni2.5Exome-8 panel, which comprehensively covers the protein content of the genome. After qual-

ity control (sex mismatch, batch effects, relatedness, chromosomal anomalies, and sample and variant quality) and missing data filters, 463 and 411 Caucasian women and men, respectively, were included in the study.

Validation was conducted in the U.K. Biobank (UKBB) cohort of 500,000 participants aged 40-69, who were recruited between 2006 and 2010 (application 20802). We used imputed genotypes for 402,000 European Caucasians, as specified in the UKBB Data Field, hereafter DF, 22006. Quality control filters consisted of heterozygosity rate (DF22010), sex mismatch, final call rate, heterozygosity outliers, unintended duplicates, and ancestry principal component outliers (DF22051). Individuals who withdrew from the study were likewise removed.

For the OPPERA cohort, the study was approved by the institutional review boards at all participating institutions. For the UKBB cohort, the study was approved by the North West Multicentre Research Ethics Committee approved the study (REC reference number: 06/MRE08/65), in accordance with the principles of the Declaration of Helsinki (information available at www.ukbiobank.ac.uk).

3.2.2 Heat QST

In the OPPERA cohort, the phenotypes analysed were measures of nociceptive summation and pain sensitivity using a heat pain wind-up protocol that preferentially stimulates C-fibres (14), as described in (15). Specifically, we queried temporal summation, a measure of the central nervous system's ability to temporally integrate the perception of noxious stimuli; and pain sensitivity, reflected by the participant's perceptual response to the first stimulus and by the area under the wind-up curve (14). Briefly, a thermode was applied to the participants' inner forearm in 3 series of 10 pulses, 1 second apart, starting at 38 °C and ending at 46 °C, 48 °C, and 50 °C, respectively. Ratings were collected from patients, on a scale from 0 to 100. The following derived measures were used as outcomes: slope of the first 3 ratings (beta, temporal summation), first and highest rating difference (delta, temporal summation), and the area under the curve (AUC, sensitivity) plotting ratings as a function of pulse count, Supplementary Figure 3.S1. The measures for 3 series amount to 12 tested phenotypes.

3.2.3 Count of reported clinical pain sites

In the UKBB, the pain phenotype used for analysis was derived from Data Field (DF) 6159, which tallied pain conditions/body sites with pain experienced at the time of assessment and for more than 3 months prior. The phenotype tested for association was the total count of pain sites, ranging from "0" to "7", and "8" corresponding to pain all over the body.

3.2.4 Hair colour

Hair colour was collected only in UKBB. Using DF 1747 we constructed the "red versus dark" phenotype, in which the "dark" category comprised dark brown and black hair colours. We excluded blonde and light brown to avoid overlap with strawberry blonde and auburn brown. The more purely dichotomous phenotype was desirable for maximum sensitivity in testing the effects of variants expected to have a comparatively small effect.

3.2.5 Statistical analyses

We ran linear regression to test for association between all genotyped MC1R variants (passing the quality control filters and minimum minor allele frequency, MAF, 1% threshold) and heat pain sensitivity and temporal summation phenotypes in OPPERA. The model of inheritance used was additive, and the baseline set of covariates used for this and subsequent analyses consisted of: TMD status, sex, age, and study site. Via David Nyholt's spectral decomposition method (https://gump.qimr.edu.au/general/daleN/SNPSpD/) (16), the starting number of variants was reduced to 8 independent genotypes. For an analogous reduction in phenotype variables we referred to the principal component analysis (PCA) of 22 heat QST measures, which output 2 independent phenotype constructs for temporal summation and pain sensivitiy, described in previous publications (14; 15). These constructs were: Component 1, with heavy loadings from temporal integration (AUC) measures for all 3 series – 46 °C, 48 °C, and 50 °C – and Component 5, with heavy loadings from temporal summation – beta and delta derived measures for all 3 series (15). The resulting correction was done for 16 tests, the product of 8 genotypes and 2 phenotypes.

Association analysis in OPPERA was done using a data storage, retrieval, and analysis portal developped by Ryan N. Lichtenwalter (17). Analysis of the UKBB data was done using Shaun Purcell's PLINK software, version 1.9 (18), and linear regression (lm) in R, version 3.5.2, was used for the analysis for association between hair colour and the count of pain conditions.

Linkage disequilibrium (LD) was visualised in Haploview software (19).

Haplotype association analysis was done using the global test in the haplo.stats package in R (20). All haplotypes above the frequency 0.01 were compared to the highest-frequency, ancestral haplotype as terms in the same multivariate model.

Expression quantitative trait locus (eQTL) effects were obtained from the genotype-tissue expression (GTEx) database (https://gtexportal.org/home/), supported by the Common Fund of the U.S. National Institutes of Health, NCI,

NHGRI, NHLBI, NIDA, NIMH, and NINDS. Summary statistics from linear regression on normalised mRNA expression levels as a function of the queried variant's minor allele count (genotype) are freely available with their GTEx eQTL Calculator tool (https://gtexportal.org/home/testyourown).

3.3 Results

3.3.1 Temporal integration and summation: discovery cohort

Single-variant association analysis was run on all genotyped *MC1R* variants with a minimum minor-allele frequency of 1%. Twelve correlated temporal summation sensitivity phenotypes at 3 temperatures were assessed (Supplementary Table 3.S1). After correcting for multiple tests (α=0.003125), 5 variants in the 5'-untranslated region (5'-UTR) of the gene were associated with the phenotype 50C AUC (sensitivity), (Table 4.1). The direction of effect was protective for all minor alleles. At the nominal significance level $\alpha = 0.05$, these 5 5'-UTR variants were associated with a number of heat QST derived measures (Supplementary Table 3.S1). LD visualisation showed all 5 variants to be members of one haploblock (Figure 3.1, Block 1), tagged by rs3212357 and rs3212361 (Table 3.2). Given that rs3212361 was the variable with the best association statistics, we tested for a possible independent contribution of rs3212357 to 50C AUC by running a multivariate regression model and found that it did not contribute above and beyond rs3212361 (P value = 0.64). Therefore for subsequent analyses, rs3212361 was retained as the sole representative of the 5' UTR variant set (Figure 3.1, Block 1), and 50C AUC was used as the phenotype best capturing the sensitivity for association with *MC1R*.

3.3.2 Association of nonsynonymous variants with pain sensitivity

Given that previous reports connected nonsynonymous MC1R variants to both red hair and pain sensitivity, next we restricted our analysis to common (minimum 1% MAF) variants known for their contribution to red hair: 6 high-penetrance, or R (rs1805006, rs11547464, rs1805007, rs1110400, rs1805008, and rs1805009) and 3 low-penetrance, or r (rs1805005, rs2228479, and rs885479) (21) for an association with 50C AUC. None of these variants passed the significance threshold adjusted for 9

Table 3.1: *MC1R* variant association analysis with 50C AUC

Variant	Minor Allele	MAF	Effect (Beta)	*P* value
rs3212361	A	0.26	-49.50	6.0e-5**
rs3212363	T	0.26	-47.41	1.0e-4**
rs3212357	G	0.42	-35.33	1.0e-4**
rs3212358	G	0.42	-34.79	2.01e-3**
rs3212354	C	0.42	-33.93	2.95e-3**

MAF, minor allele frequency. ** statistically significant association (*P* value < 3.125e-3).

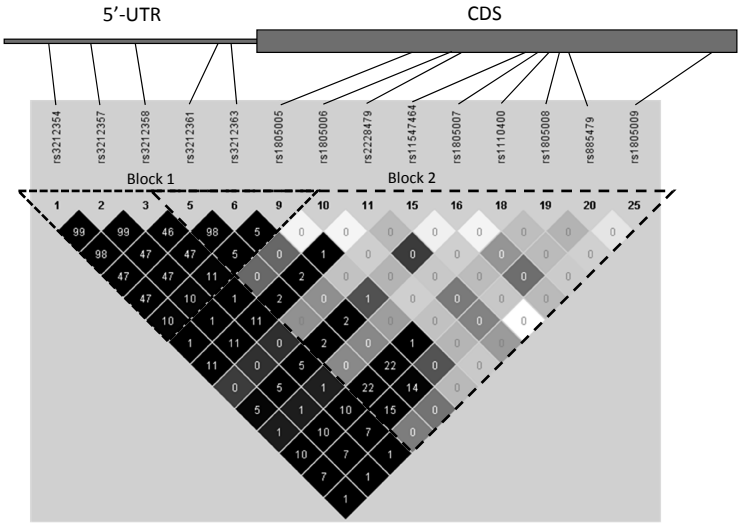

Figure 3.1: Linkage disequilibrium (LD) plot for 5'-UTR and CDS (coding region) MC1R variants. Block 1 consists of variants rs3212354, rs3212357, rs3212358, rs3212361, and rs3212363. Block 2 consists of variants rs3212361, rs1805005, rs1805006, rs2228479, rs11547464, rs1805007, rs1110400, rs1805008, rs885479, and rs1805009. Metric coding: colour = D' (white = 0; 1 < grey > 0; black = 1); values in the diamond: r-squared.

Table 3.2: Haplotypes of *MC1R* 5'-UTR variants (Block 1) above MAF 0.01

Haplotype	rs3212354	rs3212357	rs3212358	rs3212361	rs3212363	HF
1	T	A	A	G	A	5.74e-1
2	***C***	***G***	***G***	***A***	***T***	2.534e-1
3	***C***	***G***	***G***	G	A	1.667e-1

In each haplotype the minor allele is highlighted in ***boldface Italicised text***. The top haplotype is wild-type. Every other haplotype carries only one variant's minor allele. HF, haplotype frequency.

Table 3.3: *MC1R* nonsynonymous variant association analysis with 50C AUC

Variant	Minor Allele	MAF	Penetrance	Effect (Beta)	P value
rs885479	A	0.0501	r	-70.7	7.39e-3*
rs11547564	A	0.00802	R	155.2	5.11e-2
rs1110400	G	0.00772	R	65.12	2.1e-1
rs1805005	A	0.1317	r	14.25	3.79e-1
rs1805009	C	0.01722	R	35.12	3.811e-1
rs1805008	A	0.0723	R	-16.46	4.67e-1
rs1805006	A	0.00956	R	14.63	7.88e-1
rs2228479	A	0.0769	r	5.77	7.92e-1
rs1805007	A	0.0717	R	-0.222	9.92e-1

MAF, minor allele frequency. Penetrance refers to previously reported strength of correlation with red hair, R = strong and r = weak. * nominally significant association (P value < 0.05).

genotypes ($0.05/9 = 0.0083$), but rs885479 was nominally significant ($P = 0.008$), Table 3.3. An examination of all results from this analysis at the nominal significance level $\alpha = 0.05$, shows that rs885479 was associated with a number of tested measures, and rs1110400 is associated with one, Supplementary Table 3.S1.

3.3.3 Genetic correlation between 5'-UTR and nonsynonymous variants

Next we examined the LD structure between the 5'-UTR variant rs3212361 and the 9 genotyped nonsynonymous variants (rs1805005, rs1805006, rs2228479, rs11547464, rs1805007, rs1110400, rs1805008, rs885479, and rs1805009). Figure 3.1 2 (Block 2) and Table 3.4 show that 1) all nine nonsynonymous variants are in high LD (D') with rs3212361; 2) each nonsynonymous variant's minor allele appears as part of only one haplotype; 3) nonsynonymous variants' minor alleles do not co-occur; 4) only two of the nonsynonymous variants, rs885479 and rs1805008, have their minor alleles (both A) on the same haplotype with the minor allele of rs3212361, A, respectively.

Table 3.4: Haplotypes of nonsynonymous MC1R variants with 5'-UTR tag variant rs3212361 (Block 2), above HF 0.01

Haplotype	rs3212361	rs1805005	rs1805006	rs2228479	rs11547464	rs1805007	rs1110400	rs1805008	rs885479	rs1805009	HF
1	G	G	C	G	G	G	A	G	G	G	4.19e-1
2	***A***	G	C	G	G	G	A	G	G	G	1.382e-1
3	G	***T***	C	G	G	G	A	G	G	G	1.37e-1
4	G	G	C	***A***	G	G	A	G	G	G	7.2e-2
5	G	G	C	G	G	***A***	A	G	G	G	6.89e-2
6	***A***	G	C	G	G	G	A	***A***	G	G	6.75e-2
7	***A***	G	C	G	G	G	A	G	***A***	G	4.77e-2
8	G	G	C	G	G	G	A	G	G	***C***	2.088e-2
9	G	G	C	G	G	G	***G***	G	G	G	1.201e02
10	G	G	***A***	G	G	G	A	G	G	G	1.084e-2

In each haplotype the minor allele is highlighted in ***boldface Italicised text***. The top haplotype is wildtype. Every other haplotype carries only one variant's minor allele. HF, haplotype frequency.

Table 3.5: *MC1R* variant association analysis with the count of persistent pain conditions in UKBB

Variant	Minor Allele	MAF	Effect (Beta)	*P* value
1	A	2.55e-1	-8.68e-3	1.21e-2**
2	A	5.11e-2	-2.2e-2	1.54e-3**

MAF, minor allele frequency. **statistically significant association (P value < 0.025).

3.3.4 Validation cohort: count of persistent pain conditions

In an attempt to validate our findings, we analysed rs3212361 for association with the count of persistent pain conditions in the UKBB, Table 5. The minor allele of rs3212361 was indeed associated with a reduced count of pain conditions in the UKBB. We then also ran association analyses for common non-synonymous variants known for their contribution to red hair to determine if the larger sample size compared to the OPPERA cohort would identify an association. The minor alleles of rs885479, but not the other tested variants, was associated with a reduced count of pain conditions in the UKBB (Table 3.5, Supplementary Table 3.S2), reinforcing the original association pattern.

Next, we tested the variants rs3212361 and rs885479 for association with individual pain conditions, Table 3.6. The 5'-UTR variant rs3212361 was significantly associated with neck and nominally associated with back/shoulder and hip pain, for all of which its minor allele is protective. The nonsynonymous variant rs885479 was significantly associated with back/shoulder pain, and nominally associated with hip, knee, and neck pain. Thus, we identified a stable pattern of association between these variants and musculoskeletal pain, and neither variant had consistently better association statistics than the other. Given the particular haplotypic structure of MC1R, which may lead to erroneous association analysis effects in individual variant analysis (21), we investigated this further using a global haplotype association analysis on the entire set of variants in Table 3.4. The count of persistent pain conditions was used as the test phenotype. The only significant association found was for the haplotype with minor alleles at rs3212361 and rs885479 (P value $<$ 5.0e-3), Table 3.7. The next lowest P value = 5.1e-2 was for the haplotype carrying the minor allele at rs1805008 – the only other nonsynonymous variant tagging the minor allele at rs3212361. Interestingly, the haplotype carrying the minor allele at only rs3212361 was not significantly associated (P value = 2.57e-1), possibly because its weak effect was diluted when its minor allele was distributed among three separate model predictors (haplotypes 2, 5, and 6). It was not possible to run an analogous

Table 3.6: *MC1R* variant association analysis with individual persistent pain conditions in UKBB

Condition	rs3212361 Effect (OR)	P value	rs885479 Effect (OR)	P value
Back	0.98	4.38e-3*	0.955	1.67e-3**
Facial	0.997	9.24e-1	1.057	3.07e-1
Headache	1.00	9.58e-1	0.978	2.559e-1
Hip	0.974	7.29e-3*	0.961	4.18e-2*
Knee	0.99	5.54e-2	0.966	1.97e-2*
Neck	0.98	1.436e-3**	0.9691	4.05e-2*
Stomach	0.976	6.22e-2	0.9693	2.206e-1

** statistically significant association (P value < 0.00357); * nominally significant association (P value < 0.05).

haplotype association analysis in the OPPERA cohort due to an insufficient sample size for a 10-variant block.

3.3.5 eQTL analysis

Given that one of the variants associated with pain sensitivity is located in the regulatory region of the gene and the other one does not result in a strong change in cellular function phenotype (22–24), we investigated both rs3212361 and rs885479 for possible effects on gene expression using the GTEx eQTL repository. The tissues we selected to investigate were the tibial nerve, representing the peripheral nervous system, and brain structures with an established role in pain transmission or interpretation: spinal cord, amygdala, caudate, cerebellum, hippocampus, hypothalamus, putamen, anterior cingulate cortex, and nucleus accumbens. Our results show that after correcting for multiple tests (2 variants X 10 different tissues=20, P valuethreshold set at 0.05/20=0.0025) rs3212361 is significantly associated with reduced MC1R mRNA expression levels in caudate, nucleus accumbens, and tibial nerve and nominally associated with anterior cingulate cortex, cerebellum, putamen, and spinal cord, while rs885479 is only nominally associated with reduced MC1R expression in the tibial nerve, Table 3.8. Taking into account the strong LD between rs3212361 and rs885479, the association of rs885479 with *MC1R* expression in the tibial nerve is likely a reflection of the rs3212361 eQTL effect.

To determine if eQTL effects distinguished pain-associated MC1R variants from non-pain associated MC1R variants, we also extracted eQTL summary statistics for all nonsynonymous variants from Table 3.4. Supplementary Table 3.S3 shows all results with P value < 0.05 . After correcting for 80 tests (8 variants X 10 tissues), rs2228479 is a significant eQTL in all but one brain tissue (hippocampus),

Table 3.7: Haplotype association for Block 2 variants with the count of persistent pain conditions in UKBB

Haplotype	rs3212361	rs1805005	rs1805006	rs2228479	rs11547464	rs1805007	rs1110400	rs1805008	rs885479	rs1805009	HF	Effect (Beta)	*P* value
1	G	***T***	C	G	G	C	T	C	G	G	1.198e-1	4.84e-4	9.19e-1
2	***A***	G	C	G	G	C	T	C	G	G	1.193e-1	-5.44e-3	2.57e-1
3	G	G	C	G	G	***T***	T	C	G	G	9.88e-2	-6.58e-3	1.99e-1
4	G	G	C	***A***	G	C	T	C	G	G	9.58e-2	-6.28e-3	2.27e-1
5	***A***	G	C	G	G	C	T	***T***	G	G	8.38e-2	-1.07e-2	5.1e-2
6	***A***	G	C	G	G	C	T	C	***A***	G	4.98e-2	-2.76	<5.0e-3**
7	G	G	C	G	G	C	T	C	G	***C***	2.161e-2	7.58e-3	4.56e-1
8	G	G	***A***	G	G	C	T	C	G	G	1.257e-2	-1.611e-3	9.01e-1
9	G	G	C	G	G	C	***C***	C	G	G	1.132e-2	-2.11e-2	1.24e-1
10	G	G	C	G	***A***	C	T	C	G	G	6.93e-3	-1.02e-2	5.54e-1

In each haplotype the minor allele is highlighted in ***boldface Italicised text***. HF, haplotype frequency. The highest-frequency haplotype is used as the reference for the above analysis and is not shown in the table. The association statistics effect size and *P* valueare provided for analysis with the count of persistent pain conditions in UKBB. ** statistically significant association (P value < 0.005). HF, haplotype frequency.

Table 3.8: eQTL analysis: rs3212361 effects on MC1R transcript levels in peripheral and central nervous systems

	rs3212361		rs885479	
Tissue	Effect (Beta)	P value	Effect (Beta)	P value
Amygdala	-0.25	7.2e-2	-0.31	2.6e-1
Anterior cingulate cortex	-0.25	3.9e-2*	-0.27	3.1e-1
Caudate	-0.26	3.6e-4**	-0.27	1.2e-1
Cerebellum	-0.21	1.8e-3**	0.025	8.8e-1
Hippocampus	-0.15	2.5e-1	-0.043	8.7e-1
Hypothalamus	-0.13	2.3e-1	-0.11	6.9e-1
Nucleus accumbens	-0.25	3.9e-4**	-0.35	5.3e-2
Putamen	-0.24	7.2e-3	-0.24	2.7e-1
Spinal cord	-0.36	9.3e-3*	-0.087	7.5e-1
Tibial nerve	-0.17	1.2e-5**	-0.21	1.6e-2*

**statistically significant association (P value < 0.005); *nominally significant association (P value < 0.05).

however its minor allele is associated with increased levels of the MC1R transcript. Importantly, the minor allele of rs2228479 tags the major allele at rs3212361 (Table 4, haplotype 4) and has the opposite direction of effect from the minor allele of rs3212361 (Table 7). However, it is not the only marker of this major allele among the analysed nonsynonymous *MC1R* variants. The GTEx eQTL Calculator tool does not provide for haplotype association analysis. Therefore, we have not been able to test joint effects of rs3212361 and rs885479 on MC1R mRNA levels.

3.3.6 The effect of the 5'-UTR variant on red hair

Like the coding region variants associated with the red hair phenotype, which have been determined to result in a hypofunctional MC1R in cellular assays, the overall effect of the 5'-UTR region variant is also a reduction in the availability of functional MC1R. As such, we would expect it to correlate with the red hair phenotype as well. This is borne out by our analysis, which shows that the minor allele A of rs3212361 is positively correlated with red hair ($OR = 1.33$, P value = 2.0e-16).

3.3.7 The effect of red hair on pain

Lastly, we ran a regression analysis to test for association between hair colour (red versus dark) and the count of persistent clinical pain conditions. The binary-coded phenotype, red hair = 0, dark hair = 1, showed a positive association with the count of pain conditions (Beta = 4.078e-2, P value = 5.39e-5), meaning that dark hair conferred a higher risk of – and by extent red hair was protective against – having more pain conditions.

3.4 Discussion

In this study we tested *MC1R* genetic variants for association with pain sensitivity. Unexpectedly, we found that regulatory region variants but not nonsynonymous variants in this gene locus appear to affect pain sensitivity. Specifically, we found that LOF variants rs3212361 and rs885479 are associated with centrally mediated thermal nociceptive integration phenotypes. Temporal summation is a manifestation of increased firing from spinal cord dorsal horn neurons upon frequent repetitive stimulation (25; 26), demonstrated to be mediated specifically by NMDA receptors (27) and mitigated by GABA agonists (28). Temporal integration, measured by the area under the temporal summation protocol curve, has also been shown to be centrally mediated (11). Both types of summary measures reflect central processing initiated in the dorsal horn and are likely to be facilitated by reduced descending inhibition and enhanced descending facilitation (29–32). We were furthermore able to validate these findings in the UKBB, using the count of persistent clinical pain conditions as a proxy phenotype for nociceptive integration as well as the clinically relevant assessment of central sensitisation. We found that the same variants were associated with the number of reported persistent clinical pain conditions and with individual musculoskeletal pain conditions. Generally, both variants showed similar association statistics. This observation was supported by the results of our haplotype analysis, which showed the only tested haplotype with significant association be with minor alleles at rs3212361 and rs885479 (P value $<$ 5.0e-3).

The motivation behind this study lay in reconciling the previously published, apparently contradictory findings about *MC1R* variants and pain sensitivity. With access to the much larger OPPERA cohort, we set out to elucidate the effect, if any, of *MC1R* genotypes on this phenotype. Our results show that in the *MC1R* locus, contribution to pain sensitivity may stem from the 5'-UTR region, tagged by rs3212361, and from one low-penetrance red-hair variant, rs885479. The explanation for the divergent results earlier reported by Mogil's and Liem's groups possibly lies in the haplotypic structure of *MC1R*'s coding region variants, associated with red hair, and the 5'-UTR region variant, associated with pain sensitivity. Given the distribution of "red hair" alleles on the pain-associated variant's background and taking into account the relevant allele frequencies, the odds of a redhaired individual to be less sensitive to pain are about 50/50, with a slight bias toward reduced sensitivity. Of the 4 studies, 3 report genotypes for their redhaired individuals. In

Mogil's 2003 publication 8 of 14 (57 %) and in the later publication 20 of 29 (69 %) carried either the pain-protective haplotype with minor alleles at both rs885479 (and by extension rs3212361) or just at rs3212361 as tagged by rs1805008 (5; 6). In Liem's 2004 study, 4 of 10 (40 %) had at least one minor allele at either rs1805008 or rs885749 (7). Thus, while in both Mogil's studies, the participants were enriched for the pain-protective 5'-UTR region genotype, in Liem's 2004 study they were enriched for the complementary pain-relevant genotype.

The difference in the functional roles of the two relevant regions in *MC1R* invites a number of possible explanations. First, all the variants associated with red hair that have been characterised in cellular assays have been determined to compromise the cAMP-mediated activity but not *MC1R* mRNA or protein expression (23; 24; 33). Our eQTL query suggests that MC1R transcript levels are reduced in all relevant tissues by the 5'-UTR variant. Thus, downregulation of the *MC1R* mRNA appears to primarily affect pain sensitivity, while cAMP-dependent protein function appears to primarily affect hair colour. It is therefore possible that although MC1R does not directly participate in nociception, a change in its expression and/or translation levels may be trigger a feedback loop response that affects nociception or its inhibition. Four of the tissues in which rs3212361 is a significantly associated eQTL – tibial nerve, cerebellum, putamen, and caudate – suggest a role for *MC1R* in ascending nociceptive transmission (34; 35). The last associated tissue, nucleus accumbens, additionally indicates a possible involvement in affective modulation of the pain experience (36).

The nonsynonymous variant, rs885479, is not an eQTL in the tested tissues although it is nominally associated with mRNA expression in the tibial nerve. However, its reported mild cellular functional effects on reduction in ligand-binding, cell surface expression and cAMP production (23; 24) together with the effects of rs3212361 on mRNA expression would lead to a reduction in the activity of MC1R. It is unclear how this reduction in activity is different from an analogous combination of LOF alleles on haplotype 5 (Table 3.7), which carries the minor alleles of rs3212361 and rs1805008. Nevertheless, while both rs885479 and rs1805008 are LOF variants tagging the minor allele of rs3212361, a quantitative difference in their phenotype effects has already been established in the case of red hair penetrance (21).

Another explanation for our results is that the effect on pain sensitivity is exerted not by the individual minor alleles of variants rs3212361 and rs885479 but by the haplotype with both. The minor alleles of each of the two contributing variants may interact to produce a novel functional effect, for example, altering the secondary structure of the MC1R transcript and thereby compromising its stability

(37). Alternatively, rs3212361 and rs885479 may be markers for the true causal variant or region, yet to be found. Overall, the evidence available to-date, including our present findings, suggests that while MC1R does not directly participate in nociception, a reduction in its expression levels, especially when coupled with mild cellular functional impairment may modulate the nociceptive response or its inhibition.

Interestingly, while with one exception we did not find nonsynonymous red hair-associated variants to contribute to pain sensitivity, the pain-associated 5'-UTR variants, tagged by rs3212361, do contribute to red hair colour. This is consistent with the findings reported previously, which showed one 5'-UTR variant (rs76337330) in the parsimonious mRMR classifier-selected set of 10 best red hair colour predictors and 7 5'-UTR variants (rs148003355, rs3212359, rs3212361, rs3212371, rs3212379, rs577907985, and rs868197501) LASSO-selected set for a more complete genetic variant prediction model for red hair [48]. Notably, the latter model included variant rs3212361. Furthermore, when we tested binary hair colour (red versus dark) with the count of persistent pain conditions, we found a significant association between these two phenotypes, an effect possibly mediated by 5'-UTR variants: redheads showed a lower risk of reporting multiple persistent pain conditions.

It is important to recognise a number of limitations in this study. Arguably the biggest limitation is the lack of equivalent phenotypes in our discovery and validation cohorts. QST protocols are rare to be performed in large population studies, because they are time-consuming and require specialised equipment. In lieu of temporal integration and summation measures, which assess the response to repeated stimuli and which are strongly indicative of central sensitisation (11), we resorted to testing in UKBB the count of clinical pain conditions as a proxy for nociception and measure of central sensitisation. Importantly, we have shown that MC1R variants rs3212361 and rs885479 affect both experimental measures of central sensitisation and the presence of clinical conditions, in line with the reported correlation of these two phenotypes (38). Numerous studies in patients with fibromyalgia (27; 39; 40), chronic low back pain (41; 42), hip osteoarthritis (43), migraine (44), whiplash (45; 46), and TMD (11) have shown differences in temporal summation between patients and pain-free controls, with fibromyalgia, TMD, and whiplash patients showing greater pain intensity AUC. Furthermore, the larger UKBB sample size enabled us to test and verify the corresponding haplotype association with a clinically relevant outcome. However, our results should be replicated in other cohorts with heat temporal integration and summation measures and possibly other QSTs. Additional limitations of this study consist of an inability to test the joint effect of the two identified variants in the OPPERA cohort and in the eQTL dataset.

In conclusion, we report here that *MC1R* genetic variants relevant to pain sensitivity are distinct from red hair-conferring genetic variants. Our finding builds upon our understanding of the genetic architecture of human pain sensitivity and has important implications for clinical practice. As variants rs3212361 and rs885479 identified in our study to be robustly associated with the presence of multiple human pain conditions, they could be a useful inclusion into a panel of genetic contributors in pain sensitivity profiling.

3.5 Acknowledgments

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3.6 Supplementary Material

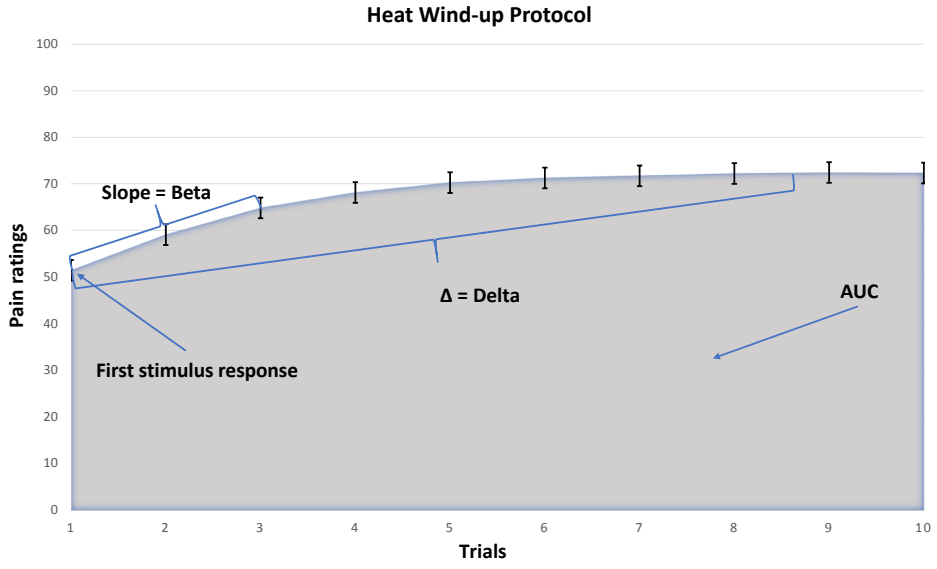

Figure 3.S1: Averaged series of pain ratings as a function of thermal stimuli (trials). The following heat pain temporal summation and sensitivity measures are shown: first stimulus response; AUC, area under the curve; beta, slope of the first 3 ratings; delta, the difference between the highest rating and the first rating. The error bars represent a 95% confidence interval.

Table 3.S1: MC1R variant association analysis with the wind-up protocol measures in OPPERA

Phenotype	Variant	Function	Minor Allele	MAF	Effect (Beta)	*P* value
50C AUC	rs3212361	5'-UTR	A	2.566e-1	-51.19	6.0e-5**
50C AUC	rs3212363	5'-UTR	T	2.587e-1	-49.84	1.0e-4**
50C AUC	rs3212357	5'-UTR	G	5.769e-1	-35.33	1.72e-3**
50C AUC	rs3212358	5'-UTR	G	5.753e-1	-34.79	2.01e-3**
50C AUC	rs3212354	5'-UTR	C	5.784e-1	-33.93	2.95e-3**
50C Delta	rs3212361	5'-UTR	A	2.566e-1	-2.56	7.28e-3*
50C AUC	rs885479	NS	A	5.006e-2	-70.70	7.39e-3*
50C Delta	rs3212363	5'-UTR	T	2.587e-1	-2.439	1.032e-2*
46C Delta	rs3212363	5'-UTR	T	2.587e-1	-1.984	1.828e-2*
48C AUC	rs3212361	5'-UTR	A	2.566e-1	-24.58	2.082e-2*
48C Delta	rs3212361	5'-UTR	A	2.566e-1	-2.051	2.14e-2*
46C Delta	rs3212361	5'-UTR	A	2.566e-1	-1.932	2.189e-2*
48C AUC	rs3212363	5'-UTR	T	2.566e-1	-3.473	2.866e-2*
48C Delta	rs3212363	5'-UTR	T	2.587e-1	-1.93	3.001e-2*
48C Beta	rs200050206	NS	A	1.54e-3	8.093e-2	3.968e-2*
48C AUC	rs885479	NS	A	5.006e-2	-44.24	4.044e-2*
48C Delta	rs1110400	NS	G	7.72e-3	8.856	4.169e-2*
48C Delta	rs3212358	5'-UTR	G	5.75e-1	-1.572	4.356e-2*
46C AUC	rs3212361	5'-UTR	A	2.566e-1	-19.29	4.532e-2*
46C AUC	rs3212363	5'-UTR	T	2.587e-1	-19.05	4.729e-2*
46C Delta	rs3212358	5'-UTR	G	5.753e-1	-1.464	4.778e-2*
46C AUC	rs885479	NS	A	5.006e-2	-38.90	4.965e-2*

MAF, minor allele frequency. MAF, minor allele frequency. All results of association analysis with *P* value < 5.0e-2 are shown. **statistically significant association (*P* value < 3.125e-3); nominally significant association (*P* value < 5.0e-2).

Table 3.S2: MC1R nonsynonymous variant association analysis with the count of persistent pain conditions in UKBB

Variant	Minor Allele	MAF	Effect (Beta)	*P* value
rs1805005	T	1.20e-1	8.26e-3	6.87e-2
rs1805006	A	1.25e-2	4.02e-3	7.59e-1
rs2228479	A	9.53e-2	-7.87e-4	8.75e-1
rs11547464	A	6.99e-3	3.17e-3	8.57e-1
rs1805007	T	9.78e-2	-3.93e-4	9.35e-1
rs1110400	C	1.17e-2	-9.24e-3	4.97e-1
rs1805008	T	8.36e-2	-9.03e-3	8.28e-2
rs885479	A	5.11e-2	-2.15e-2	1.756e-3*
rs1805009	C	2.12e-2	1.54e-2	1.30e-1

MAF, minor allele frequency. *nominally significant association (*P* value < 5.e-2).

Table 3.S3: eQTL analysis: *MC1R* nonsynonymous variant effects on transcript levels in peripheral and central nervous systems

Tissue	rs1805005 Effect (Beta)	*P* value	rs2228479 Effect (Beta)	*P* value	rs1805007 Effect (Beta)	*P* value	rs1805008 Effect (Beta)	*P* value
Amygdala	0.14	4.20e-1	0.73	2.9e-5**	-0.65	7.20e-3*	-0.075	7.20e-1
ACC	0.13	4.30e-1	0.66	1.20e-5**	-0.19	1.20e-1	-0.27	1.60e-1
Caudate	0.02	4.30e-1	0.37	3.30e-4**	-0.38	4.00e-3*	-0.3	1.20e-2*
Cerebellum	0.33	9.10e-2	0.44	6.20e-6**	0.048	7.20e-1	-0.27	1.50e-2*
Hippocampus	-0.071	6.70e-1	0.54	1.60e-3**	-0.56	7.90e-3*	0.18	3.70e-1
Hypothalamus	0.14	3.30e-1	0.56	4.10e-5**	-0.53	4.50e-3*	-0.29	8.40e-2
NAc	0.063	5.20e-1	0.43	1.10e-5**	-0.19	1.10e-1	-0.27	1.30e-2*
Putamen	0.0059	9.60e-1	0.56	2.10e-5**	-0.31	9.00e-2	-0.23	1.80e-1
Spinal cord	-0.011	9.50e-1	0.61	2.50e-4**	-0.055	8.00e-1	-0.21	3.90e-1
Tibial nerve	0.0071	9.00e-1	0.23	7.90e-5**	0.096	1.70e-1	-0.14	5.80e-2

ACC, anterior cingulate cortex; NAc, nucleus accumbens; **statistically significant association (*P* value < 5.0e-4); *nominally significant association (*P* value < 5.0e-2); NaN (not a number) for variants with the minor allele frequency < 0.01.

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Linking statement to Chapter 4

As Chapter 3 does for *MC1R* variants, Chapter 4 tests the contribution of *P2RX7* common nonsynonymous variants to pain phenotypes. Given that cellular effects of all 17 common nonsynonymous variants in *P2RX7* had not been published previously, we report their cellular functional effects in calcium imaging and patch clamp assays, prior to running statistical analyses to determine possible associations with chronic pain conditions for each individual variant. Lastly, in order to address the original hypothesis outlined in the introductory chapter of this thesis, the *P2RX7* variants with concordant functional effects in our two cellular assays are collapsed into one predictive variable using the like-effect minor allele sum to test for a possible gain of power over individual variant association.

4

Genetic variants in P2RX7 and their role in pain

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Author contributions: K.Z.-L. performed mutagenesis on the human *P2RX7* gene clone in a plasmid donated by P.S., conducted calcium imaging assays, and performed all genetic association analyses in consultation with L.D., who also reviewed and edited the manuscript. V.V.N. carried out transfection for patch clamp assays and A.R.A. performed patch clamp assays. All cellular functional assays were carried out in the lab of P.S., under his consultation.

Keywords: pain genetics, genetic variants, single-nucleotide variants, pain sensitivity, *P2RX7*, OPPERA

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Purinergic receptor-7 is encoded by *P2RX7*, a highly polymorphic gene enriched for nonsynonymous common variants. Several of these have been previously characterised for functional cellular effects and found to have either a loss-of-function (LOF) phenotype or gain-of-function (GOF) phenotype. *P2RX7* variants have likewise been implicated in modulating chronic pain risk. In this study, we undertook to functionally characterise all common (minimum minor allele frequency 1 %) nonsynonymous variants using calcium imaging and whole cell patch clamp assays, to gain additional evidence for previously reported variants' role in chronic pain, and to discover novel associations for heretofore untested variants. Among the 5 newly functionally characterised variants, rs10160951 showed a GOF in both assays, rs16950860 showed a LOF in both assays, and rs28360459, rs74357548, and rs28360460 gave mixed results. For the association analyses, we used both individual *P2RX7* variants as

predictors in univariate models and a cumulative allele count summed based on the direction of cellular effect across all tested variants with concordant cellular assay results. Association analyses were conducted in the Orofacian Pain: Prospective Evaluation and Risk Assessment (OPPERA) cohort and tested for validation in the Complex Persistent Pain Conditions (CPPC) cohorts. Our results showed an association between the LOF allele A of rs7958311 and an increased risk of chronic pelvic pain with a nominal association in the same direction for irritable bowel syndrome (IBS) in OPPERA, the latter finding validated in the CPPC cohort by same-direction associations for rs7958311 with IBS and migraine. Combined analysis did not yield better association statistics than individual variant association analyses. In conclusion, we report a comprehensive functional characterisation of *P2RX7* variant effects on ion current flow for all 17 common nonsynonymous *P2RX7* variants, including 5 previously uncharacterised variants – rs10160951, rs28360459, rs74257548, rs16950860, and rs28360460. We additionally report an association between the LOF minor allele of rs7958311 and an increased risk of chronic pelvic pain and IBS, with an association in the same direction with migraine in the validation cohort.

4.1 Introduction

Purinergic receptor 7 has an important role in the immune system response, specifically under critic conditions. This is evidenced by its low sensitivity to ATP, which allows it to respond only to ATP in excess of 100 µM. Its bimodal response manifests as an initial cation channel opening, followed by the formation of a large pore, either within the P2X7 receptor itself (1) or by recruitment of a neighbouring pannexin-1 (PANX1) (2; 3). While the involvement of P2X7 in the proinflammatory response has been extensively characterised (4), only a handful of publications have reported on its role in pain. Among the earliest reports of the involvement of P2X7 in pain comes from an animal study, which showed that this receptor's antagonist attenuated pain in an acute inflammatory pain rat model (5). A subsequent mouse P2RX7 knock-out model confirmed an abolished hypersensitivity in induced inflammatory pain and neuropathic conditions in P2RX7-null animals (6). Mice with partial nerve ligation, another neuropathic model, also showed abolished sensitivity in another study (7). Pharmacological blocking of this receptor also led

to a reduction in hyperalgesia and/or inflammation (8–10). Conversely, levels were found to be upregulated in microglia and astrocytes of bone cancer-harbouring rats, whose pain was alleviated by RNAi knock-down of (11).

The P2X7 receptor is encoded by *P2RX7*, a highly polymorphic gene with over 130 single nucleotide variants. A number of common variants have been found and examined in genetic association studies as well as functional assays. Among these, 2 GOF variants have been reported – rs208294 and rs17525809 (12) – and 7 LOF variants – rs7958311, rs2230911, rs28360447, rs1653624, rs7958316, rs28360457, rs3751143 (13–15). The cellular effects for variants rs2230912 and rs1718119 (12) have been less straitforward, with different cellular phenotypes being tested in different studies and showing different directions of functional effect (16–19).

Three of these tested variants – 1 GOF, 1 LOF, and 1 mixed – have been reported to be associated with clinical pain phenotypes. GOF variant rs208294 has been found to increase pain in post-mastectomy patients, while LOF variant rs7958311 has been found in the same cohort and in osteoarthritis patients reporting less pain (7). GOF variant rs208294 and mixed-phenotype rs1718119 have likewise shown correlation with higher pain scores in diabetic neuropathic patients (19) and rs7958311 with experimental cold pain and with clinical pain (7-day post-operative pain intensity and multisite chronic pain) (20). The question about the possible effects of other P2RX7 variants with the same or similar cellular phenotype remains unresolved, possibly due to their small effect sizes given the available sample size.

In this study we aimed to test the complete set of nonsynonymous variants with a minimum minor allele frequency (MAF) of 1% for association with a range of clinical chronic pain conditions. We first functionally characterised the common nonsynonymous variants that have been reported but not yet studied in the cellular assays. We then grouped them by direction of association before analysing each group as a single predictor. We tested chronic pain conditions available in the OPPERA cohort that had previously reported involvement from either in human association studies or in vivo animal model studies: chronic pelvic pain (21), irritable bowel syndrome (22), migraine headaches (23), and osteoarthritis (7). For validation, we used an independent CPPC cohort, which gave us access to chronic pain conditions that overlapped with the ones available in OPPERA.

4.2 Methods

4.2.1 Mutagenesis

We introduced the alternate allele of every variant into a clone of the human gene, generously donated by Dr. Philippe Séguéla, in pcDNA3.1, a mammalian expression vector, using the site-directed mutagenesis kit Q5 from New England Biolabs (Ipswich, MA, USA). The mutated constructs were purified using the BioBasic plasmid purification kit (Markham, ON, Canada) and Sanger-sequenced for verification at the McGill University and Génome Québec Innovation Centre (Montreal, QC, Canada).

4.2.2 Fluorescence imaging

In order to characterise the common non-synonymous genetic variants of P2RX7, they were transfected into human embryonic kidney (HEK293) cells using the Polyfect protocol from Qiagen (Valencia, CA, USA) on top of cells previously seeded one day prior at 300,000 per dish in 35mm polypropylene dishes from Eppendorf (Mississauga, ON, Canada). On the day following transfection, the cells were trypsinized, replated at 200,000 cells per dish on 35mm glass-bottom, poly-D-lysine coated dishes from MatTek (Ashland, MA, USA), and left overnight at 37 °C, in 5% CO2. On the following day, each dish was loaded with fura-2AM from Invitrogen (Grand Island, NY, USA) in extra cellular fluid (ECF), pH 7.4 in mM: 140 NaCl, 5 KCl, 2 CaCl2, 2 MgCl2, 10 HEPES and 10 glucose, with 0.1% BSA for 30-45 minutes at 5 mM in a 37 °C, 5% CO2 incubator. Then the dish was rinsed, fluid replaced with clean ECF without BSA, and the dish was put into the incubator for 15-30 minutes longer to remove residual fura-2AM. The dish was then mounted on a Carl Zeiss Axiovert, inverted wide-field microscope equipped with a Xenon UV lamp (Göttingen, Deutschland) and recorded for 5-6 minutes taking the ratio of 340/380 nm excitation frequencies, emission 511 nm channel intensities. Synthetic, potent P2X7 agonist, 2'(3')-O-(4-Benzoylbenzoyl)-ATP, BzATP from Sigma-Aldrich (Oakville, ON, Canada) was applied at 100 mM concentration in magnesium-free ECF for 20 seconds starting at 60 seconds using a gravity-flow perfusion system (AutoMate, Berkeley, CA, USA). After 180 seconds, 20 mM ATP (Sigma-Aldrich) was applied as a positive control, also for 20 seconds. The ATP concentration was not sufficient to activate P2X7 but was sufficient to activate other purinoceptors to verify the calcium response competence of the cells. Each variant was assayed in triplicate, starting with transfection.

4.2.3 Electrophysiology

Whole-cell patch-clamp recording of stably or transiently transfected HEK293 cells ($V_{hold} = -60\,\text{mV}$) were performed using pipettes filled with internal solution, pH 7.2, containing (in mM): 120 K-gluconate, 1 MgCl2, 5 EGTA and 10 HEPES. The recording solution, pH 7.4, comprised (in mM): 140 NaCl, 5 KCl, 2 CaCl2, 2 MgCl2, 10 HEPES and 10 glucose. Membrane currents were recorded using an Axopatch 200B amplifier and digitized at 500 Hz with a Digidata 1330 interface (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA). Only recordings with series resistance below 10 MΩ and stable for the duration of the recording were considered for analysis. Drugs were dissolved in recording solution and applied using a SF-77B fast perfusion system (Warner Instruments, Hamden, CT, USA) at a rate of 1 ml/min. All experiments were performed at 22 °C. For each individual experiment, current amplitudes were compared and expressed as a percentage.

4.2.4 OPPERA cohort

The discovery cohort for this study was drawn from the Orofacial Pain Prospective Evaluation and Risk Assessment (OPPERA) project, designed and implemented according to the methods layed out in (24). 1883 participants, 1400 women and 908 men, passed genotyping sample quality filters. Of these participants, 705 were diagnosed cases of temporomandibular disorder (TMD) upon recruitment. Genotyping was done at 2.5 million exonic single variant positions on the Illumina Infinium Omni2.5Exome-8 panel, which comprehensively covers the protein content of the genome.

The participants in the OPPERA study were also queried for the presence of chronic pain conditions. Among them, we selected the conditions in which P2RX7 has a likely role, based on previous reports of genetic association and/or animal model studies: migraine, osteoarthritis, rheumatoid arthritis, IBS, and chronic pelvic pain.

4.2.5 CPPC cohort

The second replication cohort was part of the Complex Persistent Pain Conditions (CPPC): Unique and Shared Pathways of Vulnerability study, conducted at the University of North Carolina, Chapel Hill, North Carolina, U.S.A. This study of overlapping pain conditions recruited and genotyped 800 participants with at least one of four index CPPCs: episodic migraine, irritable bowel syndrome, fibromyalgia, and vulvar vestibulitis. Subjects were aged 18-64, and included both sexes (86% female) and major ethnic and racial groups (68% non-Hispanic white).

4.2.6 Genetic association analysis

We ran linear regression to test for association between all common nonsynonymous *P2RX7* variants (passing the quality control filters and minimum minor allele frequency, MAF, 1% threshold) and chronic pain phenotypes in OPPERA. The model of inheritance used was additive, and the baseline set of covariates used for this and subsequent analyses consisted of: TMD status, sex, age, and study site. Via David Nyholt's spectral decomposition method (https://gump.qimr.edu.au/general/daleN/SNPSpD/) (25), the starting number of variants was reduced to 16 independent genotypes. We used 4 phenotypes: chronic pelvic pain, migraine, osteoarthritis, and IBS. The resulting correction was done for 64 tests, the product of 16 genotypes and 4 phenotypes.

Association analysis in OPPERA was done using a data storage, retrieval, and analysis portal developped by Ryan N. Lichtenwalter (26). Analysis of the CPPC data was done using Shaun Purcell's PLINK software, version 1.9 (27).

4.3 Results

4.3.1 Functional characterisation

Seventeen common nonsynonymous variants were characterised for functional effects on P2RX7 using two assays, Table 4.1, Figure 4.1 and Figure 4.2. The calcium imaging assay measured intracellular calcium upregulation in response to P2RX7 stimulation by BzATP. Of 17 variants, 5 showed a gain-of-function (GOF) cellular phenotype, 7 showed loss-of-function (LOF), and the remaining 5 showed no effect. In the patch clamp assay, 4 showed a GOF effect, 9 showed a LOF effect, and the remaining 4 no effect. Six of the characterised variants showed concordance between the two assays: rs208294, rs7958311, rs28360447, rs10160951, rs16950860, and rs7958316. Among these, rs208294, rs7958311, rs28360447, and rs7958316 have been both previously characterised and also show concordance in the reported direction of cellular functional effect for the minor allele.

4.3.2 Genetic association

The top result for the association study with clinical pain conditions was for chronic pelvic pain, associated with variant rs7958311 (OR=2.518, P value = 5.0e-5), Table 4.2. This was also the only result passing multiple testing correction. However,

Table 4.1: *P2RX7* functional effects

Variant	Minor Allele	MAF	Published	Ca2+	Patch clamp
rs208294	A	0.4239	GOF	GOF	GOF
rs7958311	A	0.2562	LOF	LOF	LOF
rs2230911	C	0.103	LOF	GOF	LOF
rs28360447	A	0.1272	LOF	LOF	LOF
rs1653624	A	0.0161	LOF	No effect	No effect
rs10160951	C	0.03763	N/A	GOF	GOF
rs28360459	A	0.02526	N/A	GOF	LOF
rs74357548	A	0.001	N/A	LOF	No effect
rs16950860	A	0.00699	N/A	LOF	LOF
rs7958316	A	0.01523	LOF	LOF	LOF
rs28360457	A	0.01021	LOF	No effect	LOF
rs28360460	A	0.00520	N/A	No effect	LOF
rs2230913	G	0.03299	No effect	No effect	GOF
rs17525809	G	0.0570	GOF	LOF	GOF
rs2230912	G	0.1242	mixed	LOF	No effect
rs1718119	A	0.391	mixed	GOF	No effect
rs3751143	C	0.1744	LOF	No effect	LOF

MAF, minor allele frequency. **statistically significant association (P value $<$ 3.125e-3). Column 3 (Published) has lists previously reported functional associations, where available, and N/A for uncharacterised variants. Columns 4 and 5 list our findings from the calcium imaging and patch clamp assays, respectively.

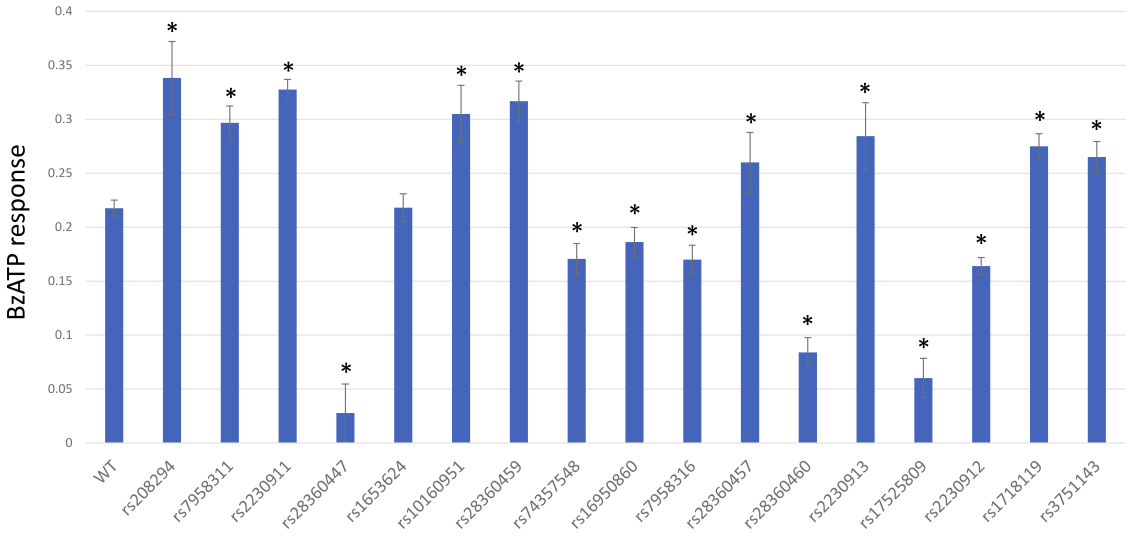
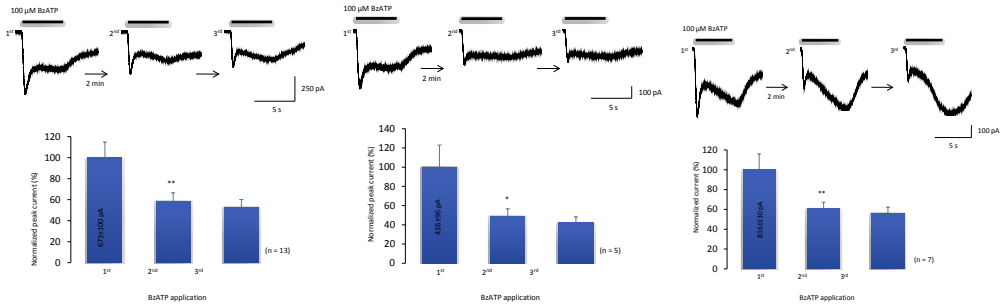


Figure 4.1: Calcium imaging results quantification for *P2RX7* variants. The error bars represent 95% confidence intervals, and the stars (*) denote a statistically significant (P value $<$ 0.05) T-test comparison with wildtype.



(a) Wildtype (b) LOF (c) GOF

Figure 4.2: Patch clamp results quantification for *P2RX7* variants.

Table 4.2: *P2RX7* variant association analysis with temporal summation

Phenotype	Variant	Minor Allele	Functional effect	MAF	Effect (OR)	*P* value
Chronic pain pelvis	rs7958311	A	LOF	0.2562	2.518	5.0e-5**
IBS	rs7958316	A	LOF	0.01523	2.707	2.07e-3*
IBS	rs7958311	A	LOF	0.2562	1.365	1.635e-2*
Osteoarthritis	rs208294	A	GOF	0.423	0.598	3.324e-2*
Chronic pain pelvis	rs7958316	A	LOF	0.01523	3.102	4.276e-2*
IBS	rs16950860	A	LOF	0.00699	2.756	4.846e-2*

MAF, minor allele frequency. *nominally significant association (P value $<$ 5.0e-2); **statistically significant association (P value $<$ 7.81e-4).

as is also true for the two other LOF variants rs7958316 and rs16950860, all the associations for rs7958311 are risk for the minor allele. GOF variant rs208294 is associated with osteoarthritis in the protective direction.

We next conducted combined analysis for the top associated phenotype, chronic pelvic pain, using as genotypic predictor the summed count of minor alleles for the two GOF variants (rs208294 and rs10160951) with the major allele count of LOF variants (rs7958311, rs28360447, rs16950860 and rs7958316). The results were once again not significant (P value = 0.0974).

Validation: CPPC

We furthermore attempted to validate the clinical pain findings in OPPERA in the CPPC cohort. The relevant pain conditions assessed in this cohort were IBS and migraine, both of which were significantly associated with rs7958311 in the same direction as in OPPERA, i.e. the minor allele conferred risk, Table 4.3.

Table 4.3: Validation association analysis in CPPC

SNP ID	Phenotype	Effect Allele	Effect (OR)	p-value
rs7958311	A	IBS	1.566	3.154e-3**
rs7958311	A	Migraine	1.445	1.048e-2**

IBS, irritable bowel syndrome; effect size is listed as "OR", odds ratio; *nominally significant association (P value < 5.0e-2); **statistically significant association (P value < 2.5e-2).

4.4 Discussion

Here we report the cellular functional characterisation of all common nonsynonymous variants in P2RX7 in calcium imaging and patch clamp assays as well as genetic association for individual variant alleles and for a combined genotype obtained by summing all like-effect variant alleles.

Based on our findings in the calcium imaging and patch clamp assays, *P2RX7* harbours 2 GOF variants – rs208294 and rs10160951, 4 LOF variants – rs7958311, rs28360447, rs16950860, and rs7958316, 1 with no effect in either assay – rs1653624, and 10 with discordant phenotypes between the two assays – rs2230911, rs28360459, rs74357548, rs28360457, rs28360460, rs2230913, rs17525809, rs2230912, rs1718119, and rs3751143. Our assays showed concordance with previously reported functional effects for 1 GOF variant: rs208294 (17; 18); and 3 LOF variants: rs7958311, rs28360447, rs7958316 (28). Among previously uncharacterised variants, our two assays showed the same direction of functional effect for GOF rs10160951 and LOF rs16950860. Additionally, three more previously uncharacterised variants – rs28360459, rs74357548, and rs28360460 – showed mixed cellular effects in calcium imaging and patch clamp.

The lack of congruence between the two assays for some variants is puzzling. One possible explanation would be the variants' effects on *P2X7*'s different modes of activity. Not all cellular functions attributed to P2X7 are mediated through calcium influx. For example, P2X7's pore-opening has been reported in numerous studies as a calcium-influx independent activity (29–31). Thus, it is possible that a given variant could change the receptor's calcium flow mediation while its other modes of signalling that impact upon whole-cell current flow – perhaps through protein-protein interactions – remain intact or are altered in a different manner.

Our genetic association finding showed an increased risk for chronic pelvic pain and a nominal association with an increased risk for IBS correlated with the minor

allele of LOF variant rs7958311 in the discovery cohort – OPPERA. This finding was validated in the replication cohort, CPPC, with a statistically significant association with IBS. An association in the same direction for the minor allele of rs7958311 was also found for migraine in CPPC, although it was not even nominally associated with this condition in OPPERA. These results contrast with previously reported associations for the LOF minor allele of this variant with reduced pain in postmastectomy and osteoarthritic patients (7), in neuropathic pain patients (19), as well as with reduced response to cold pain stimulation (20). A possible explanation for this divergence in the direction of effect is the difference in etiology. Previously reported protective association for rs7958311 was with conditions that have a strong neuropathic pain component (32). The associations reported here fall into two other pathophysiological categories. Chronic pelvic pain, which is an umbrella term for a number of conditions characterised by pain localised in the pelvic region, often has an inflammatory component and includes and IBS – another inflammatory condition – as a possible cause (33; 34). Migraine, in turn, has a neurovascular pathophysiology (35).

Analysis using combined variant alleles by direction of their cellular functional effect did not yield better association statistics compared to individual variant analysis. Thus, the additive effect of all tested P2RX7 variants does not outweigh the effect of the top associated variant. Instead, additional variants are likely to only contribute noise.

In conclusion, we report here the functional cellular phenotypes of all 17 common nonsynonymous P2RX7 variants, 5 of which have not been previously characterised in cellular assays to our knowledge. We further report findings in support of the minor allele of rs7958311 being a risk-increasing genetic variant for chronic pelvic pain and IBS, with a suggested association in the same direction with migraine.

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5

General Discussion and Conclusion

5.1 Discussion

The studies described in this thesis were driven by the hypothesis that genetic contribution to variability in a common phenotype, such as the pain experience in our case, is distributed among many common variants with minute effects and can be better detected in a statistical association test by summing their like-effect allele counts into one predictive variable. Allelic effects were determined using cellular functional assays or drawn from the literature reporting the results of such assays, prior to summing their counts. If evidence in support of this hypothesis had been found, it would have offered a more robust method of quantifying the overall cellular function-informed effect of multiple contributing variants in a particular genetic locus.

The idea of combining variants for added statistical power to detect a genetic association with a phenotype is not novel. Several methods have been published to test the cumulative effects of multiple variants, including single nucleotide polymorphism (SNP)-set test, burden tests, and nonburden SKAT. The SNP-set test groups variants based on functional genomics, such as proximity to genes or haplotype blocks (1). Burden tests are powerful in regions with many causal, same-direction and similar-effect magnitude variants (2). One specific type of burden test assigns a risk score – a weighted sum of regression coefficients from single-variant genetic association tests – which is used as the predictor for the phenotype of interest (3). Nonburden sequence kernel association tests (SKAT) are variance component tests, powerful in regions with a large number of noncausal variants or variants with different directions of effect (2; 4). However, all these tools use variants as the intermediaries to connect the tested phenotype to the genetic region they represest, meaning that the goal behind the design of cumulative-analysis methods has been to identify genes contributing to a given phenotype (5).

With some exceptions – namely the SNP-set test, which includes functional ge-

nomic annotation, and *in silico* cellular effect prediction (6) – cumulative tests have been combining variants grouped or weighted based on statistically derived discriminators. Our approach was instead to annotate variants by the direction of their cellular functional effect and group them accordingly. This variant-centric approach aimed to improve the detection of correlation between the overall cellular functional effet of a given genetic locus and the clinical phenotype effect. Furthermore, rather than making provisions for rare variants as is done in the other cumulative tests, we set the frequency threshold at 1% and excluded all variants with lower minor allele frequencies.

The genes selected for our study – *MC1R* and *P2RX7* – were taken from a list of pain-relevant loci with a high density of common nonsynonymous variants reported in human studies to be associated with pain phenotypes (7). Both genes had been determined to affect the pain experience in a mouse model and reported to have associations with human pain phenotypes (8–10).

Additionally, the nonsynonymous variants in *MC1R* had a robust association with red hair, which served as a training phenotype for defining a functional genetic construct for this locus. Due to the difference in functional regions within *MC1R* – 5'-UTR versus CDS – that were most relevant to pain and red hair, this functional construct did not also capture the genetic effects of *MC1R* on pain sensitivity. Nevertheless, it was useful in identifying this difference and in revealing the singular haplotypic structure of this locus, namely the correlations between the pain-relevant 5'-UTR and red hair colour-relevant CDS regions. Furthermore, *P2RX7* variants, which had both loss- and gain-of-function cellular phenotypes, permitted us to query a locus with possible compensatory mechanisms.

The following assumptions were made. A top-down view of genetic effects on pain presents pain processing as dependent on a large number of pathways, whose protein mediators are encoded by their respective genes, which may harbour functional single-nucleotide variants, where functional means having a quantifiable effect on the resulting protein's activity. Given that the functional effect of each variant is either to reduce or enhance the protein's activity, their overall contribution should be additive. The first assumption is therefore that the overall effect of the gene on the pain phenotype is a sum of effects of the variants harboured within its locus. The second assumption is that regardless of the specific affected cellular phenotype, the effect of each variant corresponds to an overall reduction or enhancement of the protein's activity.

With these assumptions in mind, we grouped allele counts based on the direction of their cellular effect with the intention to improve the statistical power of associ-

ation with a complex phenotype without increasing the sample size. Only common variants, minimum 1% minor allele frequency in the population, were included, to ensure that our OPPERA subsample sizes of 800 and 1800 for the two genes, *MC1R* and *P2RX7*, respectively, were sufficient to detect the expected small effects.

In the case of *MC1R*, summing the minor allele count of all nonsynonymous variants (rs1805005, rs1805006, rs2228479, rs11547464, rs1805007, rs1110400, rs1805008, rs885479, and rs1805009) determined in previously-published cellular assays to have the same direction of functional effect – loss of activity – when analysed with the top associated phenotype, area under the heat wind-up curve (AUC), yielded the association statistics: Beta = 3.26, P value = 0.78 , compared to the top result in individual variant analysis: Beta = -70.7, P value = 0.0074 .

The loss of statistically significant association in the summed test is likely due to the haplotype distribution of the relevant variant alleles. Given that the main region associated with heat wind-up AUC is the 5'-UTR (Table 1, Chapter 3), and that all nonsynonymous variants (except for rs885479) do not have a significant individual association with this phenotype (Table 3, Chapter 3), their direction of effect would be driven by the allele of the 5'-UTR variant, rs32123161 on their haplotypes. And given that only rs885479 (which has its own pain-protective association, Table 3, Chapter 3) and rs1805008 minor alleles mark the minor, pain-protective allele of rs3212361 (Table 4, Chapter 3), the majority of the variant alleles summed mark the complementary allele of rs3212361, which confers no protection against pain sensitivity.

The likeliest reason that *MC1R* combined analysis did not yield better association statistics than individual analysis is that only one of the summed variants – rs885479 – contributes to the tested phenotype. The other variants, despite having the same direction of cellular effect as rs885479, do not appear to affect heat wind-up AUC. Furthermore, while the top variant allele that is statistically associated with heat wind-up AUC – rs3212361 – is correlated with reduced transcript levels of MC1R in pain-relevant nervous system tissues (Table 8, Chapter 3) and by extension could lead to reduced activity in the protein MC1R, like the hypofunctional nonsynonymous variants, it does not share its effects on pain sensitivity with the coding region variants.

In the case of *P2RX7*, summing like-effect alleles of the nonsynonymous variants with the same cellular phenotype direction of effect as determined in our calcium and patch clamp studies meant adding the major alleles of two gain-of-function variants rs208294 (G) and rs10160951 (G) – with the minor alleles of four loss-of-function variants rs7958311 (A), rs7958316 (A), rs28360447 (A), rs16950860 (A) – yielded

the following association statistics for chronic pelvic pain: OR = 1.49, P value = 0.0053 , compared to the top result in individual variant analysis: OR = 2.51, P value = 0.00006 .

While combining *P2RX7*'s like-effect variant alleles yields a significant P value, the OR is a lower magnitude than in individual variant analysis. The likeliest explanation is that some of the summed alleles have sufficiently dissimilar contributions to the pathway responsible for affecting the phenotype in question that their individual effects add more noise than signal to the joint variant effect. There are two known modes of action in *P2RX7* – pore and cation influx, and pore forming is responsible for association with pain. The variant locations are also of note – rs208294, rs28360447, rs7958311, rs7958316, and rs16950860 are in the extracellular loop, and rs10160951 is in the intracellular carboxyl terminus, which is reported to be involved in forming the pore (10).

The findings reported in this study reveal the need to look beyond the gene and focus on individual variants, treating them as multidimensional and interdependent contributors to whole-person phenotypes. The knowledge acquired in the field of human genetics since the sequencing of the genome in 2001 (11) has countered the temptation to classify genetic variants as exclusively deleterious, or function-reducing/abolishing. Gain of function and compensatory epistasis – or variant interactions that mitigate their individual effects – have expanded our understanding of genetic variant impact beyond functional impairment and have redrawn for us these effects on a bidirectional plane. The present thesis, however, has shown that this view, limited to overall gain and loss may still not be representative of the complete picture of genetic variant functional effects.

Both *MC1R* and *P2RX7* have variant effects that segregate by location, presumably tied to the pathway in which they participate. For *MC1R*, pain-affecting variants appear to be in the regulatory 5'-UTR, and their effects impact upon transcript levels, which we propose may affect the pro-opioid production upsteam of *MC1R*. The red hair-affecting variants lie in the coding region of *MC1R*, which implies that changes in the protein composition affect pigment production. In *P2RX7*, the two cellular assays show that sometimes effects on cation flow and pore opening are disparate, and differences downstream of these pathways may be responsible for differences in phenotype effects. *TRPV1*, the third gene that had originally been included in this study, and for which the association with pain phenotypes is described in Appendix C, has also shown similar cellular phenotype-specific effects. Variant rs222747 (M315I) in the amino-terminal domain of the *TRPV1* receptor displayed descreased current whole-cell current flow but increased calcium permeation (12).

While the primary outcome was to explore a potential gain in statistical power by combining like-effect allele counts, the secondary outcome was to bring into question the utility of designations "loss of function" and "gain of function", currently in widespread use in genetic association literature. The two examples of *MC1R* and *P2RX7* show that a single gene-wide assignment of overall gain or loss of function may not be informative for deciphering their effects on common phenotypes, and further cellular-phenotype and consequently pathway-specific granularity is requisite.

Furthermore, as we have shown in the MC1R study detailed in Chapter 2, from the perspective of statistical association analysis, the direction of individual variant effects given likely multi-locus effects should be considered in the context of their haplotypic structure and relative magnitude of effect. Specifically, highly correlated variants with widely disparate effect sizes may yield flipped directions of effect in individual-variant analysis, thereby further confusing the interpretation of GOF and LOF variant effects.

A variant-focused approach in future genetic association studies means treating each variant as the unit of effect on a clinical phenotype. A bottom-up approach would mean starting with all likely functional variants, i.e. nonsynonymous. Variant characterisation would be conducted more thoroughly and multidimensionally, using a panel of cellular assays for testing all likely downstream effects. In addition, eQTL effects should be assessed statistically. Gain- and loss-of-function labels should be assigned based on its effects on each assay individually and on eQTL association effects. However, focusing on each variant should not be mistaken for treating it as existing in isolation. Haplotype structure should be considered to determine possible compensatory or additive effects of multiple functional variants on a haplotype (13; 14).

In addition to assessing the utility of combined variant analysis, a third outcome of the studies described in this thesis was an expansion upon the current understanding of the role of genetic variants in *MC1R* and *P2RX7* in pain phenotypes. Aside from animal studies on analgesic effects of α-MSH injections into the PAG (15) and proalgesic effects of this hormone, also believed to antagonise opioid receptors (16), Mogil and Liem have put forward a number of suggested mechanisms whereby MC1R and its main agonist α-MSH could be related to the pain response.

Mogil's two studies have shown that Mc1r double-null mice have better responses to opioid analgesics acting on κ- and μ-opioid receptors (8; 9). In their first publication, they suggest that given MC1R's nanomolar affinity for κ-opioid ligands – such as its endogenous agonist dynorphin (17) – this opioid-melanocortin activity

may counter analgesic effects of an opioid ligand binding its own receptor (8). An analogous MC1R-mediated anti-μ-opioid receptor activity is proposed in the second publication, in which the μ-opioid receptor agonist produces stronger analgesia in both mice and humans (9).

Liem's two publications offer two other possible explanations. First, the authors suggest that the main responsible melanocortin receptor may be MC4R, which is widely expressed in the CNS and has a similar affinity for α-MSH as MC1R (18). MC4R's antagonist alleviates cold and mechanical allodynia in rats with neuropathic pain, which is reversed by μ-opioid receptor's antagonist, naloxone (16). Functional antagonism between the melanocortin and opioid systems is furthermore evidenced by colocalisation of the respective receptors in the CNS (19). Second, pituitary functions are mainly controlled by negative feedback systems, as shown by α-MSH injections into the paraventricular hypothalamic nucleus that decrease *POMC* gene expression (20). Hypofunctional MC1R may therefore upregulate α-MSH production, whose MC4R- or opioid receptor- mediated proalgesic activity increasing baseline pain sensitivity (18; 21).

Our own finding of statistical association between the 5'-UTR transcript-level reducing *MC1R* variant and reduced pain sensitivity suggests another possibility for the feedback loop referenced in Liem's 2004 publication. Instead of (or perhaps in addition to) a hypofunctional MC1R signalling for an upregulation of proalgesic α-MSH, reduced *MC1R* transcript levels may signal for an upregulation of another POMC-derived peptide, β-endorphin, the endogenous μ-opioid receptor ligand with analgesic effects. Higher levels of β-endorphin could explain lower baseline sensitivity to experimental heat pain, for which we report statistically-derived evidence in Chapter 3.

As a multi-faceted alarm-sounding immune system agent, P2X7 has been demonstrated to have protective as well as pathology-promoting roles, depending on the level of its activation, cell type, pathogen type, and infection severity (22). Its activity culminates in the activation of an inflammasome, which leads to a large-scale release of proinflammatory cytokines and proalgesic neurotransmitters. Just like acute pain, which serves a protective function, may turn into a chronic debilitating condition if it outlasts the duration of its initial trigger, acute inflammation in response to pathogen invasion or cell damage may turn into a chronic debilitating condition if left unresolved. Evidence for its involvement in neuropathic and inflammatory pain conditions exists from both rodent and human studies (10; 23). The effects of P2X7 as determined by statistical association and mouse genetic studies pointed to its pore-opening function as pro-algesic (10). In our study (Chapter 4),

a pore-mediating variant is statistically significantly associated with visceral pain. Given that visceral chronic pain conditions may be sequelae of unresolved infections (24), it is possible that a hypofunctional P2X7 permits excessive infection or infection-related damage to affected tissues due to an inadequate intial response (25).

5.2 Conclusion

In this work, we attempted to exploit same-direction cellular functional effects of common nonsynonymous genetic variants to gain statistical power in demonstrating association. This was done by combining all small-effect variant alleles within a particular genetic locus based on the direction of their functional effects as determined by cellular assays. While this methodological approach did not improve association statistics for the selected variants in our two selected genes, it did lead to a comprehensive examination of all common nonsynonymous variants in MC1R and P2RX7, which resulted in several important conclusions applicable to these genes and to the human genome broadly.

Gene-specific findings are the following. In the MC1R red hair study (Chapter 2), we have shown that although both weak-effect and strong-effect nonsynonymous variants contribute to red hair, conventional single-variant analysis results in a statistical reversal of the direction of effect. Our study furthermore confirmed that each variant previously reported to be associated with red hair did in fact contribute to red hair. Additionally, *MC1R* genetic variants did not distinguish between non-red hair colours, and other genes did not contribute significantly to red hair colour, above and beyond *MC1R*. This work also resulted in a predictive model with an area under the receiver operating characteristic curve (AUROC) performance metric of 0.95 for an economical 10-variant model and an AUROC of 0.96 for a larger LASSO-selected variant model.

In the *MC1R* pain sensitivity study (Chapter 3), we have shown that the variants contributing to pain in this gene locus are primarily located in the 5'UTR, tagged by the variant rs3212361, while the main region contributing to red hair is *MC1R*'s coding region. Nevertheless, additional contribution was detectable from one low-penetrance red hair variant – rs885479. Furthermore, haplotype analysis suggested nonadditive multi-locus effects. These analyses have shown that *MC1R* variants may contribute to both heat pain sensitivity – baseline response to noxious

thermal stimuli – and heat temporal summation – or increased dorsal horn neuronal excitability upon repeated stimulation of a given frequency. The validation study of these associations has also shown a possible contribution to the count of persistent clinical pain conditions as a proxy for central sensitisation.

The *P2RX7* study (Chapter 4) has shown that the previously reported pain-protective LOF variant rs7958311 may contribute to a higher risk of visceral inflammatory pain conditions and migraine. Functional cellular characterisation of the complete panel of common nonsynonymous variants has shown that the variants may affect calcium influx and whole-cell electrical currents differently.

On the genome scale, study outcomes are the following. First, cellular functional phenotype is not always the best predictor of a clinical phenotype, and caution should be exercised before assuming that variants with quantitatively similar *in vitro* assay outcomes would have the same clinical phenotype outcomes. Second, the relative magnitude of effects in combination with an LD structure that leads to mutually exclusive presence of variants may lead to the flipped direction of effect in single-variant association analysis. Third, genetic locus pleiotropy may lead to an erroneous assumption that the same variants in the given locus affect all associated phenotypes. Fourth, clinical phenotypes may be affected differently depending on the region of the gene locus harbouring them, i.e. coding region variants may affect one phenotype, while regulatory-region variants in the same gene may affect another. Although function-impairing amino acid substitution and transcript downregulation may both result in reduced availability of the functional protein, their exact means of reducing this availability may have important implications for downstream phenotypes.

In conclusion, genetic association studies of pain conditions continue to provide essential insights into the highly complex network of molecular mechanisms that contribute to pain processing, sensitivity, and chronicity. The work presented in this thesis is an appeal to increase the resolution of genetic contribution from gene-level to sub-gene single-base pair scale. While the current standard in genetic association studies is to consider each variant in a gene as a representative of its locus' effect on a given phenotype, we have shown here that grouping variants by direction of cellular effect as well as ascribing pleiotropy to the same sub-gene region without a careful gene-wide characterisation and consideration of effect size and LD structure may lead to erroneous results.

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6
Abbreviations

179InsC, 179-insert-cytosine (frameshift mutation)
5-HT, 5-hydroxytryptamine
5'-UTR, 5'-untranslated region
A188G, alanine-188-glycine
ACAN, aggrecan
ACE, angiotensin I converting enzyme
ACTH, adrenocorticotropic hormone
α-MSH (α-melanocyte stimulating hormone)
ADAMTSL4, a disintegrin and metalloproteinase with thrombospondin motifs-like 4
ADARB2, adenosine deaminase RNA specific B2
ADRA1D, adrenoceptor alpha 1D
ADRA2C, adrenoceptor alpha 2C
ADRB2, adrenoceptor beta 2
AJAP1, adrehens junctions associated protein 1
ANKK1, ankyrin repeat and kinase domain containing 1
APOA1BP, apolipoprotein A-I binding protein
APOE, apolipoprotein E
AR, androgen receptor
ARMS2, age-related maculopathy susceptibility 2
ASIP, agouti-signalling protein
ASTN2, astrotactin 2
ATP, adenosine triphosphate
ATP1A2, ATPase Na+/K+ transporting subunit alpha 2
ATP5B, ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit
AUROC, area under the receiver-operating characteristic curve
BDNF, brain derived neurotrophic factor

BSA, blood serum albumin
BzATP, 2'(3')-O-(4-Benzoylbenzoyl)-adenosine triphosphate
C7orf10, succinyl-CoA:glutarate-CoA transferase
Ca2+, calcium
CaCl, calcium chloride
CACNA1A, calcium voltage-gated channel subunit alpha1 A
CAMK4, calcium/calmodulin dependent protein kinase IV
CARF, calcium responsive transcription factor
CASP1, caspase-1
CASP9, caspase 9
cAMP, cycle adenosine monophosphate
CCI, chronic constriction injury
CCM2L, cerebral cavernous malformation 2-like scaffold protein
CCR2, C-C motif chemokine receptor 2
CCT5, chaperonin containing TCP1 subunit 5
cDNA, complementary deoxyribonucleic acid
CDS, coding region
CERC, Canada Excellence Research Chair
CFDP1, craniofacial development protein 1
CFTR, cystic fibrosis transmembrane conductance regulator
CGRP, calcitonin gene-related peptide
CHRM2, cholinergic receptor muscarinic 2
CO2, carbon dioxide
COMT, catechol-O-methyltransferase
CPPC, complex persistent pain conditions
CPQ, carboxypeptidase Q
CRHBP, corticotropin releasing hormone binding protein
CXCL8, C-X-C motif chemokine ligand 8
D294H, aspartic acid-294-histamine
D84E, aspartic acid-84-glutamic acid
DAO, D-amino acid oxidase
dbGAP, genotypes and phenotypes database
DBH, dopamine beta-hydroxylase
dbSNP, single nucleotide polymorphism database
DF, datafield
DNA, deoxyribonucleic acid
DOCK4, dedicator of cytokinesis 4

DOPA, dihydroxyphenylalanine
DRD2, dopamine receptor D2
DRD4, dopamine receptor D4
DRG, dorsal root ganglion
ECF, extracellular fluid
EDNRA, endothelin receptor type A
EGTA, egtazic acid
ESR1, estrogen receptor 1
ESR2, estrogen receptor 2
eQTL, expression quantitative trait locus
EXOC2, exocyst complex component 2
FAM183B, family with sequence similarity 183 member B
FANCA, FA Complementation Group A gene
FDR, false discovery rate
FGF6, fibroblast growth factor 6
FHL5, four and a half LIM domains 5
FKBP5, FKBP prolyl isomerase 5
FSHR, follicle stimulating hormone receptor
FUT9, fucosyltransferase 9
GABA, gamma-aminobutyric acid
GABRB3, gamma-aminobutyric acid type A receptor beta3 subunit
GBP1, guanylate binding protein 1
GCH1, GTP cyclohydrolase 1
GDF, gain de fonction
GDF5, growth differentiation factor 5
GDNF, glial cell-derived neurotrophic factor
GJA1, gap junction protein alpha 1
GLM, generalized linear model
GOF, gain of function
GPCR, G protein-coupled receptor
GPK2, G protein kinase 2
GRK5, G protein-coupled receptor kinase 5
GPK6, G protein kinase 6
GPR149, G protein-coupled receptor 149
GRCh37, Genome Reference Consortium Human genome build 37
GRIA1, glutamate ionotropic receptor AMPA type subunit 1
GRIA3, glutamate ionotropic receptor AMPA type subunit 3

GSTM1, glutathione S-transferase mu 1
GTEx, gene-tissue expression project
GTP, guanosine triphosphate
GWAS, genome-wide association study
HCRTR1, hypocretin receptor 1
HF, haplotype frequency
HEK293, human embryonic kidney cells (a cell line)
HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HERC2, HECT and RLD domain containing E3 ubiquitin protein ligase 2
HEY2, hairy-enhancer-of-split related family bHLH transcription factor with YRPW motif 2
HIV, human immunodeficiency virus
HLA, human leukocyte antigen
HLA-DRB1, major histocompatibility complex, class II, DR beta 1
HPA axis, hypothalamic-pituitary-adrenal axis
HPSE2, heparanase 2
HTR2A, 5-hydroxytryptamine receptor 2A
HTR7, 5-hydroxytryptamine receptor 7
I155T, isoleucine-155-threonine
I585V, isoleucine-585-valine
IASP, International Association for the Study of Pain
IBS, inflammatory bowel syndrome
IGSF9B, immunoglobulin superfamily member 9B
IFRD1, interferon related developmental regulator 1
IL1A, interleukin 1 α
IL1β, interleukin-1β
IL1R1, interleukin 1 receptor type 1
IL1R2, interleukin 1 receptor type 2
IL1RN, interleukin 1 receptor antagonist
IL9, interleukin 9
IL10, interleukin 10
IL10RB, interleukin 10 receptor subunit beta
IL13, interleukin 13
IL18R1, interleukin 18 receptor 1
IL18RAP, interleukin 18 receptor accessory protein
INSR, insulin receptor
IRF4, interferon regulatory factor 4

ITPK1, inositol-tetrakisphosphate 1-kinase
JAG1, jagged canonical Notch ligand 1
KCl, potassium chloride
KCNAB3, potassium voltage-gated channel subfamily A regulatory beta subunit 3
KCNG4, potassium voltage-gated channel modifier subfamily G member 4
KCNK18, potassium two pore domain channel subfamily K member 18
KCNS1, potassium voltage-gated channel modifier subfamily S member 1
KITLG, KIT ligand
LASSO, least absolute shrinkage and selection operator
LD, linkage disequilibrium
LDLR, low density lipoprotein receptor
LOF, loss of function
LRP1, LDL receptor related protein 1
LRRIQ3, leucine rich repeats and IQ motif containing 3
LTA, lymphotoxin alpha
M315I, methionine-315-isoleucine
MAF, minor allele frequency
MAOA, monoamine oxidase A
MC1R, melanocortin-1 receptor
MC2R, melanocortin 2 receptor
MC4R, melanocrotin-4 receptor
MED14, mediator complex subunit 14
MEF2D, myocyte enhancer factor 2D
MgCl2, magnesium chloride
MITF, microphthalmia-associated transcription factor
MLR, multiple linear regression
MMP1, matrix metallopeptidase 1
MMP2, matrix metallopeptidase 2
MMP3, matrix metallopeptidase 3
MMP16, matrix metallopeptidase 16
MNSOD, superoxide dismutase 2
MPPED2, metallophosphoesterase domain containing 2
mRMR, minimum redundancy maximum relevance
mRNA, messenger ribonucleic acid
MRVI1, murine retrovirus integration site 1 homolog
MTDH, metadherin
MTHFD1, methylenetetrahydrofolate dehydrogenase, cyclohydrolase and formylte-

trahydrofolate synthetase 1
MTHFR, methylenetetrahydrofolate reductase
MTRR, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase
MYT1L, myelin transcription factor 1 like
N29insA, asparagine-29-insert-adenine (frameshift mutation)
NA, not available
NaCl, sodium chloride
NaV, voltage-gated sodium channel
NCBI, National Center for Biotechnology Information
ND, not determined
NGFR, nerve growth factor receptor
NHGRI, National Human Genome Research Institute
NHLBI, National Heart, Lung, and Blood Institute
NIDA, National Institute on Drug Abuse
NIH, National Institute of Environmental Health Science
NIMH, National Institute of Mental Health
NINDS, National Institute of Neurological Disorders and Stroke
NFKBIA, NFKB inhibitor alpha
NMDA, N-methyl-D-aspartate
NOS3, nitric oxide synthase 3
NOTCH3, notch receptor 3
NOTCH4, notch receptor 4
NPV, negative predictive value
NR3C1, nuclear receptor subfamily 3 group C member 1
NRP1, neuropilin 1
NRXN3, neurexin 3
NTRK1, neurotrophic receptor tyrosine kinase 1
OCA2, OCA2 melanosomal transmembrane protein
OPPERA, Orofacial Pain: Prospective Evaludation and Risk Assessment (study)
OPRM1, opioid receptor μ1
OR, odds ratio
P91S, proline-91-serine
P2RX7, purinergic receptor 7
PAG, periaqueductal grey
PANX1, pannexin-1
PC, principal component
PCA, principal component analysis

PDF, perte de function
PGK1, phosphoglycerate kinase 1
PGR, progesterone receptor
PHACTR1, phosphatase and actin regulator
PLCE1, phospholipase C epsilon 1
POMC, pro-opiomelanocortin
PPV, positive predictive value
PubMed, reference database for publications and abstracts on lifesciences and biomedical topics
PRDM16, PR/SET domain 16
PRRT2, proline rich transmembrane protein 2
PRSS1, serine protease 1
PTGS2, prostaglandin-endoperoxide synthase 2
Q85R, glutamine-85-arginine
R142H, arginine-142-histamine
R151C, arginine-151-cysteine
R160W, arginine-160-tryptophan
R163Q, arginine-163-glutamine
RAMP1, receptor activity modifying protein 1
REST, RE1 silencing transcription factor
RH, red hair
RHC, red hair colour
ROC, receiver operating characteristic
RNAi, ribonucleic acid interference
RNF213, ring finger protein 213
RUNX2, RUNX family transcription factor 2
QIMR, Brisbane Twin Nevus Study, Australian Twin Registry, and Tasmanian Eye Study
RS, Rotterdam Study
QQ, quantile-quantile (plot)
QST, quantitative sensory testing
SCN1A, sodium voltage-gated channel alpha subunit 1
SCN9A, sodium voltage-gated channel alpha subunit 9
SCN10A, sodium voltage-gated channel alpha subunit 10
SCN11A, sodium voltage-gated channel alpha subunit 11
SERPINA6, serpin family A member 6
SHMT1, serine hydroxymethyltransferase 1

SIFT, sorting intolerant from tolerant (algorithm)
SKAT, sequence kernel association test
SLC24A3, solute carrier family 24 member 3
SLC24A4, solute carrier family 24 member 4
SLC45A2, solute carrier family 45 member 2
SLC6A4, solute carrier family 6 member 4
SMAD3, SMAD family member 3
SNI, spared nerve injury
SNP, single nucleotide polymorphism
SPINK1, serine peptidase inhibitor, Kazal type 1
STAT6, signal transducer and activator of transcription 6
T469I, tyrosine-469-isoleucine
TAAR1, trace amine associated receptor 1
TGFB1, transforming growth factor beta 1
TGFBR2, transforming growth factor beta receptor 2
TMD, temporomandibular disorder
TMJ, temporomandibular joint
TMP, transcripts per million
TNF, tumour necrosis factor-α
TNFRSF1B, TNF receptor superfamily member 1B
TPCN2, two pore segment channel 2
TPM, transcripts per million
TRPA1, transient receptor potential cation channel subfamily A member 1
TRPM8, transient receptor potential cation channel subfamily M member 8
TRPV1, transient receptor potential, vanilloid 1
TRPV3, transient receptor potential cation channel subfamily V member 3
TSPAN2, tetraspanin 2
TYR, tyrosinase
TYRP1, tyrosinase-related protein 1
UKBB, (U.K.) United Kingdom Biobank
UTR, untranslated region
UV, ultraviolet
V60L, valine-60-leucine
V92M, valine-92-methionine
V_{hold}, holding voltage
VNTR, variable number of tandem repeats
VR1, vanilloid receptor 1

WSCD1, WSC domain containing 1
Y152OCH, tyrosine-152-termination (premature protein termination)
YAP1, Yes associated protein 1
ZCCHC14, zinc finger CCHC-type containing 14

A

Appendix A: Genetic predictors of human chronic pain conditions

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Keywords: chronic pain conditions, genetic association studies, GWAS, pain genetics, single nucleotide polymorphisms

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Chronic pain conditions are multifactorial disorders with a high frequency in the population. Their pathophysiology is often unclear, and treatment is inefficient. During the last twenty years, genetic linkage analysis and association studies have made considerable strides toward identifying key molecular contributors to the onset and maintenance of chronic pain. Here, we review the genetic variants that have been implicated in chronic pain conditions, divided into the following etiologically-grouped categories: migraine, musculoskeletal pain disorders, neuropathic pain disorders, and visceral pain disorders. In rare familial monogenic pain conditions several strong-effect mutations have been identified. In contrast, the genetic landscape of common chronic pain conditions suggests minor contributions from a large number of single nucleotide polymorphisms representing different functional pathways. A comprehensive survey of up-to-date genetic association results reveals migraine and musculoskeletal pain to be the most investigated chronic pain disorders, in which nearly half of identified genetic variability alters neurotransmission pathways.

A.1 Introduction

Chronic pain is a persistent maladaptive condition, estimated to affect up to 30 % of the world's population (1). Given the reported heritability of 16 % (2; 3), a substantial proportion of the risk of developing a chronic pain condition is driven by genetic background. To date the search for contributing genetic variants has yielded an outline of a centralized pain-processing system, spearheaded by neurotransmitters and their receptors and modulated by myriad other factors, ranging from inflammatory cytokines to growth factors. Although a gestalt understanding is important for conceptualizing chronic pain, genetic studies have shown a cosegregation of distinct pathologies with their putative causal factors at the gene/protein level. Therefore, it will be helpful to present an overview of the current knowledge about genetics of chronic pain separated by etiology, known or hypothesized.

Numerous genetic risk factors have been identified for musculoskeletal, neuropathic, and visceral conditions, as well as migraine. Among these, migraine and musculoskeletal pain disorders have undergone the most extensive investigation in association studies and have accumulated the highest number of implicated genetic variants (Figure A.1), although many of them await replication (Figure A.2). The list of chronic pain genes (Table A.1) is a snapshot of the incredible complexity of the suspected network of molecular interactions. This list includes genes from catecholaminergic, serotonergic, estrogenic, glutamatergic, GABAergic, purinergic and orexinergic pathways; cytokines; growth factors; and proteinases.

The purpose of this review is threefold: 1) provide an overview of the current state of knowledge in human chronic pain genetics, 2) highlight relevant genetic studies and their outcomes for each pathology category, and 3) summarize the genomically-derived mechanisms of molecular pathophysiology for each category.

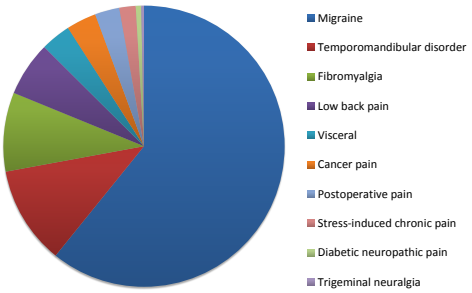
A.1.1 Genetic variability

The human genome is replete with genetic variants. The majority are germline mutations, passed from parents to offspring. Less common *de novo* mutations are not inherited from parents and occur in offspring only. Examples of *de novo* mutations in sodium channels, Nav1.7, *SCN9A*, and Nav1.9, *SCN11A*, have been described in post-trauma pain perception, congenital insensitivity to pain, and primary erythromelalgia (4; 5). Somatic mutations acquired during one's lifetime are not passed on to offspring. These mutations have been implicated in cancer but so far have not been associated with chronic pain conditions.

Rare but drastic mutations have been identified as causal in several monogenic familial disorders, in which mutations in a single gene locus result in the onset of

Condition	# of associations
Migraine	194
Temporomandibular disorder	36
Fibromyalgia	29
Low back pain	20
Visceral pain	11
Cancer pain	11
Postoperative pain	9
Stress-induced chronic pain	7
Diabetic neuropathic pain	2
Trigeminal neuralgia	1

(a)

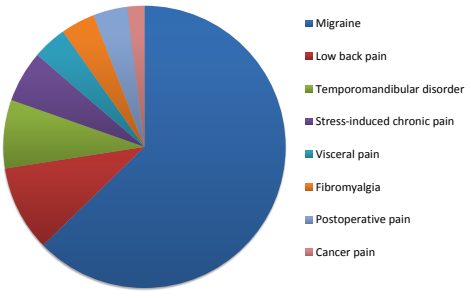


(b)

Figure A.1: **Chronic pain conditions quantified by the number of genetic association studies.** For each condition, the number of published genetic associations is given, including both positive and negative results. Rare Mendelian disorder variants from linkage studies are not included.

Condition	# of citations
Migraine	32
Low back pain	5
Temporomandibular disorder	4
Stress-induced chronic pain	2
Visceral pain	2
Fibromyalgia	2
Postoperative pain	2
Cancer pain	1

(a)



(b)

Figure A.2: **Chronic pain conditions quantified by the number of genetic loci.** Only genes with positive association in a given disorder or group of disorders reported in at least two studies are included, and rare Mendelian disorder variants from linkage studies are not included.

the condition. For example, a frameshift mutation, which severely compromises the function of the encoded protein, has been discovered in the TWIK-related spinal cord potassium channel (TRESK) gene, *KCNK18*, and is responsible for familial migraine with aura (6). A more common type of causal mutation in rare familial disorders is a nonsynonymous mutation in one nucleotide that leads to an amino acid change of substantial functional effect in the resulting protein. Sodium channels are the best-known example, extensively studied for their role in monogenic conditions such as erythromelalgia, caused by mutations in *SCN9A* (7).

Unlike the rare, high-impact mutations described above, common single nucleotide polymorphisms (SNPs), found in $> 1\,\%$ of the population, comprise the vast majority of human genetic association studies. These mutations usually have a very minor phenotypic effect and often exert their effect in concert with specific environmental pressures. Rather than directly causing a chronic pain disease, these SNPs modulate susceptibility to it. The minor allele contributes either risk or protection by increasing (conferring gain-of-function on) or decreasing (conferring loss-of-function on) the activity of the resultant protein. Approximately $90\,\%$ of SNPs are found in introns or intergenic regions, outside of the protein-coding segments of the gene, outlining their regulatory role. SNPs that fall in the exonic, or protein-coding, region may be either non-synonymous, resulting in a different amino acid, or synonymous, not changing the amino acid. Whether intronic or exonic, synonymous or non-synonymous, most common SNPs are silent and have no clearly observable or discernible phenotypic effect.

Several databases, such as NCBI dbSNP (`http://www.ncbi.nlm.nih.gov/snp`), the 1000genomes project (`http://www.1000genomes.org`), and Ensembl (`http://www.ensembl.org`), inventory known SNPs and annotate them with genomic location, population distribution, expression, and functional effect, adding disease association information where available. The recently launched Human Pain Genetics Database (`http://diatchenko.lab.mcgill.ca/hpgdb`) is a manually-curated repository for SNPs in human genes reported to be associated with pain conditions or intermediate phenotypes.

A.1.2 Human genetic studies

With few exceptions, chronic pain does not follow the Mendelian transmission model. Rather, chronic pain pathologies are typically aggregates of endophenotypes, each of which may be governed by Mendelian law. A construct developed for the study of complex diseases in neuropsychiatry, endophenotypes must 1) be heritable, 2) be associated with the disease of interest, 3) be manifest in subjects independently of

active pathology, and 4) cosegregate with disease in pedigree studies (8). In this case, endophenotypes are symptoms of chronic pain conditions. They include sleep disturbance, fatigue, depression, cognitive decline, and hypersensitivity to external stimuli.

The two predominant ways geneticists screen human subjects for mutations associated with chronic pain are linkage analysis and association studies. For linkage analysis available family members are phenotyped and genotyped to see which genetic locus or loci will segregate with the disease. This method has been used to identify a rare mutation in *KCNK18*, encoding the above-mentioned potassium channel TRESK (6). Naturally, this type of study depends on access to several generations of a pedigree. Linkage analysis is efficient for identification of rare familial mutations.

Association studies, used to identify common functional SNPs, require large populations of unrelated subjects with the pathology of interest to be matched against a population of healthy controls. Their DNA is genotyped using either a targeted selection or a genome-wide high-density panel of SNPs. Given the high number of subjects needed in order to provide sufficient statistical power, genome-wide association studies (GWAS) are usually done through collaboration of many research institutions. Results from association studies can be further combined in meta-analysis to get reliable association metrics for tested SNPs. The implementation and maturation of large consortia during the past decade have led to valuable online resources, such as NHGRI (`https://www.genome.gov`) and NCBI dbGaP (`http://ncbi.nlm.nih.gov/gap`), which offer comprehensive catalogues of GWAS results. However, to date the number of GWAS on chronic pain conditions is very limited. Study design is complicated by the lack of standardized chronic pain phenotype definitions, heterogeneity in clinical reporting, and comorbidities (9). Migraine is the exception: four GWAS (10–13) and three GWAS meta-analyses (14–16) have been reported for this condition. A fibromyalgia GWAS (17) and a GWAS meta-analysis for chronic widespread pain (18) have been recently published as well.

A.1.3 Animal Models

While human studies are limited by accessibility to subjects with the genotypes of interest, in animal models, target genotypes can be engineered and screened. The mouse is the most common model organism in pain genetics. Back-crossing and selective inbreeding are done to isolate a particular locus on a wild-type background. A gene of interest is knocked out, knocked down, or a human clone is knocked in to observe its effect on a particular pathology. Transgenic mouse models have been

reviewed in (19). Certainly, phenotype characterization is more difficult given non-verbal subjects, and genomic and physiological differences between the two species necessarily result in phenotypic discrepancies. Mouse model developers must demonstrate that their models are sufficiently similar to the human condition under study to be informative. Despite these difficulties, a number of models (20) have been very instrumental in either identifying genes involved in chronic pain conditions or confirming their relevance after identification in a human study. Neuropathic pain models include spared nerve injury (SNI), chronic constriction injury (CCI), diabetic, and cancer pain (21). Inflammatory pain models use injections of immune system stimulants, such as Freund's adjuvant and carrageenan, as well as nociceptor sensitizer brandykinin, and pro-inflammatory cytokines. Orofacial and visceral chronic pain states are modeled using site-specific injections of inflammatory agents (22). Several migraine mouse models have been likewise described. While earlier studies focused on brain structural-anatomical singularities and electrophysiology experiments in transgenic animals with alterations in causal genes (23), more recent models have endeavored to find a behavioral proxy for migraine symptoms in mice (24).

A.2 Methods

The list of studies reporting genes associated with chronic pain disorders was drawn from the Human Pain Genetics Database (http://diatchenko.lab.mcgill.ca/hpgdb). This list was supplemented with a literature search in Google Scholar for studies reporting suggested association or negative results. For each disorder, the search terms included the name of the disorder and the gene as well as one of the following terms: "genetic association", "variant", or "polymorphism". Studies of disorders in which pain is not the primary symptom, such as diabetes and cancer, were only included if the finding specifically stated association with pain. Publications were screened by title and abstract; in cases where these presented insufficient information, the text of the publication and relevant tables was read. Reviews and publications reporting duplicate results from the same cohort were excluded.

With the exception of several large populations studies, which are specified below, the majority of the studies conducted to date have been done on small population samples (fewer than 1000 individuals). Additionally, excepting four migraine GWAS (10–13), three migraine GWAS meta-analyses (14–16), one fibromyal-

gia GWAS (17), and one chronic widespread pain GWAS meta-analysis (18), all studies were candidate-gene studies or gene panel studies. While the number of GWAS and sequencing studies continues to rise, candidate gene association studies have played an important role in our understanding of the genotypic structure of human pain phenotypes. Although these hypothesis-driven studies risk oversight of untested causal genetic variants and augment the chances of false positive associations (25; 26), when replicated in multiple cohorts, they point to functional variants and the direction of their effects.

A.3 Results

A.3.1 Migraine

Migraine, a complex and debilitating pain disorder, is estimated to affect up to 25 % of women and 8 % of men (27). Similar to musculoskeletal conditions, up to 50 % of migraine etiology has been attributed to genetic factors (28). Numerous migraine genetics reviews have been published in the last decade (29–33), and the number of genes with reported statistically significant association exceeds that of all other chronic pain conditions (Figure A.1). Notably, some of these genes were identified in GWAS. Partly as a testament to its clearly defined diagnostic criteria – lacking for most other pathologies discussed here – migraine is one of the few chronic pain conditions to have undergone GWAS, of which there are four to date (10–13). The last of these was done in a genetically-isolated Norfolk Island population (13). In addition, there have been three meta-analyses (14–16), the last of which is the most comprehensive reported migraine genetic study of individuals with European ancestry, having analyzed a total of 375.000 individuals and having identified 38 loci of susceptibility, replicating 10 previously published associations.

The paradigm of migraine causality continues to shift between vascular dysregulation and neuronal hyperexcitability, as compelling evidence continues to accumulate for both theories. Genetic studies have contributed significantly to the current understanding of its molecular pathophysiology.

Familial hemiplegic migraine

Rare mutations in three genes have been reported as causal in familial hemiplegic migraine (FHM): *CACNA1A* in FHM1 (34); *ATP1A2* in FHM2 (35); and *SCN1A* in FHM3 (36). The *CACNA1A*-encoded alpha-1 subunit of a P/Q voltage-gated

calcium channel, also reported to be involved in cortical spreading depression, has gain-of-function mutations that lead to channel responsiveness at lower voltages and contribute to a state of neuronal hyperexcitability. *ATP1A1*-encoded alpha-1 subunit of the sodium-potassium ATPase pump affects its ability to pump sodium ions against the concentration gradient, necessary for glutamate and calcium flow (35). *SCN1A* encodes the alpha-1 subunit of a voltage-gated neuronal sodium channel, and its minor allele hastens the channel's recovery from fast inactivation, increasing cortical neuron firing frequency (36). Proline rich transmembrane protein 2, *PRRT2*, is a recent addition to the list. Mutations in this gene have also been implicated in hemiplegic migraine (37; 38). Not an ion channel like the other three FHM variants, PRRT2 may be involved in neuronal exocytosis and release of neurotransmitters by affecting localization and kinetics of channels such as CACNA1A (37). FHM1-3 causal variants and *PRRT2*, discovered through pedigree linkage mapping, are responsible for rare migraine disorders.

Migraine: vascular origins

Genetic association studies have also identified variants with higher frequency and lower effect, thought to be involved in the much more prevalent nonhemiplegic migraine. Unlike the FHM disorders, the hypothesized causality of the more common migraine may or may not be limited to hyper-neuroexcitation. Evidence continues to accumulate for the involvement of the originally-suspected vascular system dysregulation. According to the vascular hypothesis, cogently summarized in (39), a combination of inadequate response to oxidative stress, lower levels of vasodilators, and increased numbers of vasoconstrictors brings about endothelial dysfunction, which may lead to an elevated level of proinflammatory cytokines circulating in the extracellular matrix. Genetic variants supporting vascular dysregulation in migraine include *EDNRA*, encoding endothelin type A receptor (40; 41); *MTHFR*, encoding methylenetetrahydrofolate reductase (42–56); *NOS3*, encoding endothelial nitric oxide synthase (57; 58); *ACE*, encoding angiotensin-1 converting enzyme (43; 59–63); *NOTCH3*, encoding a receptor involved in vascular development and integrity (64; 65); *TGFB1*, encoding beta-2 transforming growth factor (58); and *TGFBR2*, encoding beta-2 transforming growth factor receptor (12; 16; 66) (which has not shown an association in a North Indian population (67)). As further evidence for the involvement of the vascular system in migraine pathophysiology, Winsvold *et al.* have recently reported a large overlap between associated loci in a multi-study migraine GWAS meta-analysis and coronary artery disease GWAS (68). Specifically, migraine without aura and coronary artery disease share significantly associated SNPs but in opposite directions.

Migraine: inflammatory markers

A vast number of genetic association studies have implicated a modified inflammatory state in migraine directly by showing a correlation between alleles of cytokines TNF-α, *TNF* (58; 69–73) (not replicated in (74) and (75)), and TNF-β, *LTA*, with migraine (70; 74–76). A tumor necrosis factor receptor superfamily member, *TNFRSF1B*, has also been implicated as a risk factor in migraine susceptibility in a Han Chinese population (77); as have interleukin 1-β, *IL1B* (71); interleukin 9, *IL9*; chemokine receptor, *CCR2* (58); and prostaglandin endoperoxide synthase 2, *PTGS2* (78; 79).

Migraine: neuronal origins

Current evidence is arguably strongest for susceptibility to migraine lying at the intersection of increased ascending nociceptive signaling and reduced descending inhibition (80). Implicated signaling systems involve glutamatergic, serotonergic, dopaminergic, GABAergic, orexinergic and purinergic transmission.

In the glutamatergic system, whose involvement in migraine has been recently reviewed in (81), AMPA receptors, *GRIA1* (82) (not found to be associated in (83) and (84)) and *GRIA3* (82; 84); glutamate receptor *GRM7* (13); as well as glutamate-regulating metahedrin, *MTDH* (10; 12; 16); lipoprotein receptor, *LRP1* (11; 16; 66; 67); myocyte enhancer factor, *MEF2D* (12; 16; 66); and a variant near plasma glutamate carboxypeptidase, *CPQ* (10) have been reported as associated with migraine. Except for the two AMPA receptors, all these genes were top hits in migraine GWAS, replicated in a later GWAS or in subsequent targeted SNP genotyping projects (12; 66).

The role of serotonergic transmission in migraine has been comprehensively reviewed in (85). Serotonin receptor, *HTR7*, has been shown to be associated in a Norfolk Island population GWAS (13). Likewise, an association with migraine has been reported for serotonin transporter, *SLC6A4*, in a Turkish population (86) and a Japanese population (87). Furthermore, Gonda *et al.* have reported an association between this locus and migraine comorbid with anxiety (88). However, *SLC6A4* has not been found to be significantly associated in a German cohort (89), which may indicate that the effect is race-specific.

Dopaminergic involvement, recently reviewed in (90), is of particular interest given its reported association not only with migraine but also with anxiety and depression (both well-established comorbidities with migraine (91; 92)). Peroutka *et al.* and Del Zompo *et al.* have discussed dopaminergic pathway-mediated changes in cerebral blood flow (observed during cortical spreading depression) and somatosen-

sory hyperactivity (93; 94), which would explain prodromal symptoms such as moodiness, drowsiness, and nausea (95; 96). Dopamine beta-hydroxylase, *DBH* (97–100); dopamine D2 receptor, *DRD2* (93; 94; 100); and dopamine D4 receptor, *DRD4* (101; 102) (which has been reported not associated in (94)) have all been implicated in migraine and some of the associated non-headache symptoms. *DRD2* has also been reported to be associated with aura, anxiety and depression (93; 94), as well as aortic stenosis, another vascular disorder (103). Cargnin *et al.* have reported on the role of dopamine-degrading catechol-*O*-methyl transferase *COMT* in the response to triptans for migraine (104).

GABAergic involvement has been demonstrated through association with a GABA-A receptor, *GABRB3* (105; 106) (not replicated in (107)). Additionally, a sodium channel, *SCN1A*, hypoactive variant has been shown to suppress the activity of GABAergic inhibitory interneurons (33).

The orexinergic system, whose suspected role in migraine has been recently reviewed in (108), is implicated through an association between migraine and hypocretin receptor 1, *HCRTR1* (109). Hypocretin is a neuropeptide that regulates arousal, wakefulness, and appetite.

The purinergic system may be involved as well, as evidenced by association for adenosine deaminase, *ADARB2* (13) and mitochondrial ATP synthase, *ATP5B* (15), both GWAS results, and purinergic receptor 7, *P2RX7*, which has so far been linked to migraine in a mouse model (110).

Further evidence for dysregulated neuronal excitability comes from associations between migraine and four synaptic plasticity mediators in GWAS: neuronal cation exchanger, *SLC24A3* (16); phosphatase and actin regulator, *PHACTR1*; astrotactin, *ASTN2* (12; 16; 66); and transcription enhancer, *FHL5* (15; 16; 66; 111). Two recent publications have announced key rare variants in the first gene to be implicated through a linkage mapping pedigree study in nonhemiplegic migraine: *KCNK18*, encoding the potassium channel TRESK (6; 112). Considering the likelihood of neuroexcitability as the main culprit, the same research group has targeted a panel of ion channels, transporters, exchangers and accessory subunits and found two more potassium channels, *KCNG4* and *KCNAB3*, to be associated with migraine (113). Likewise, (114) have reported a calcium-activated potassium ion channel, *KCNN3*, variant to be protective against migraine. Lemos *et al.* have shown a significant interaction between brain-derived neurotrophic factor, *BDNF* (also reported to be associated in (115) but not in (116)), and calcitonin gene-related peptide, *CGRP*, with migraine (117). Histamine-degrading enzyme, diamine decarboxylase, *DAO*, has likewise been reported associated in a Caucasian Spanish

population (118). A mild association has been reported for a variant in *RAMP1* – a CGRP receptor subunit – with migraine in (119) and with migraine changing into medication-overuse headache (120) (although another group has reported no significant association with medication overuse headache in migraineurs (121).

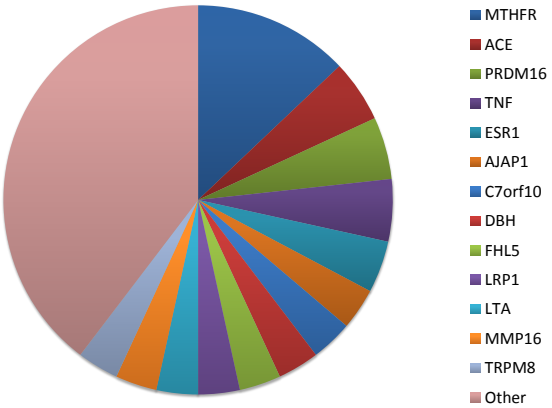
Other genes that have been identified in genetic association studies include estrogen receptors, *ESR1* (122–126) (not replicated in (127)) and *ESR2* (124) (not replicated in (125)); follicle stimulating hormone receptors *FSHR* (124); progesterone receptor, *PGR* (128) (not replicated in (127)); low-density lipoprotein, *LDLR* (129) (not replicated in (130)); and human leukocyte antigen, *HLA-DRB1* (131); insulin receptor, *INSR* (132); and ankyrin repeat and kinase domain containing 1, *ANKK1* (100). Another is *PRDM16*, whose minor allele was one of three significantly associated risk variants coming from the second migraine GWAS (the other two being *TRPM8*, discussed below, and *LRP1*, discussed above) (11). As a transcription factor involved in brown fat development, PRDM16 is a dubious suspect in terms of migraine pathology. This association has, however, been replicated in two Chinese population studies (133; 134) and two large-population European studies (16; 66). Another replication attempt has reported association in the opposite direction (as a protective variant) (67), and the same study has also reported positive replication for *LRP1*. In addition, Christensen *et al.* have reported a role for *PRDM16* in modulating migraineurs' response to treatment with triptans (135). Rubino *et al.* have reported an association for neurogenic locus notch homolog protein *NOTCH4* with migraine (136), replicated in the largest GWAS meta-analysis to date (16).

Another high-powered meta-analysis of 23 285 individuals with migraine and 95 425 controls demonstrated significant associations with adherens junction-associated protein 1, *AJAP1*; tetraspanin, *TSPAN2*; succinyl-CoA:glutarate-CoA transferase, *C7orf10*; matrix metalloproteinase 16, *MMP16*; apolipoprotein A-I binding protein, *APOA1BP*; fucosyltransferase 9, *FUT9*; interleukin 4-induced activator of transcription 6, *STAT6*; and TBC1 domain family member, *TBC1D7* (15). *AJAP1*, *MMP16* and *C7orf10* have been replicated in a Chinese population (111) and two other GWAS (16; 66), which have also replicated the association for *TSPAN2*. Nerve growth factor receptor-encoding *NGFR* has also been found associated in a six-center GWAS meta-analysis in Dutch and Icelandic populations (14). There has likewise been an association reported for a locus in the mitochondrial DNA with migraine, both with and without aura (137).

Migraine has been aptly styled "a cycle of painful signaling and interpretation of nonpainful stimuli as painful" (138). In line with the latter part of this statement, the reported contribution of primary afferent nociceptor-expressed transient

Gene	# of citations
MTHFR	15
ACE	6
PRDM16	6
TNF	6
ESR1	5
AJAP1	4
C7orf10	4
DBH	4
FHL5	4
LRP1	4
LTA	4
MMP16	4
TRPM8	4
Other	46

(a)

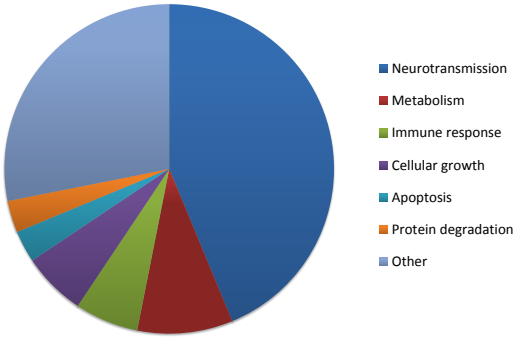


(b)

Figure A.3: **Genetic loci associated with migraine, quantified by the number of genetic association studies.** Only genes with association reported in at least two studies are included in the analysis, and only genes with association reported in at least four studies are listed individually. Genes with fewer reported associations are grouped under "Other." Abbreviations: *MTHFR*, methelynetetrahydrofolate reductase; *ACE*, angiotensin I converting enzyme; *PRDM16*, PR domain-containing 16; *TNF*, tumor necrosis factor; *ESR1*, estrogen receptor 1; *AJAP1*, adherens junctions-associated protein 1; *C7orf10*, succinyl-CoA:glutarate-CoA transferase; *DBH*, dopamine beta-hydroxylase; *FHL5*, four-and-a-half LIM domains 5; *LRP1*, low density lipoprotein receptor related protein 1; *LTA*, lymphotoxin alpha; *MMP16*, matrix metalloproteinase 16; *TRPM8*, transient receptor potential cation channel, subfamily M, member 8 (menthol and cold receptor). Rare Mendelian disorder variants from linkage studies are not included.

Condition	# of citations
Neurotransmission	14
Metabolism	3
Immune response	2
Cellular growth	2
Apoptosis	1
Protein degradation	1
Other	9

(a)



(b)

Figure A.4: **Functional pathways of genetic loci associated with migraine quantified by the number of loci.** Only genes with at least one replicated association are included. Rare Mendelian disorder variants from linkage studies are not included. For genes reported to be involved in multiple pathways, the pathway of the translated gene's most direct involvement was chosen.

receptor potential (TRP) receptors adds a central sensitization component to the pathophysiological agglomerate that leads to the migraine disorder. Light, heat, and mechanical sensors – vanilloid receptors, *TRPV1* and *TRPV3* (139), – and a cold temperature and menthol receptor, *TRPM8*, found to be associated with migraine in a number of GWAS (11; 12; 16; 66), suggest that migraineurs' pain is engendered by nociceptive response to physiologically-innocuous stimuli.

Migraine genetics: summary

Among chronic pain conditions, genetic studies of migraine are the most abundant (Figure A.2) and have output at least 30 different genes with replicated association (Figure A.3). The most highly cited gene is *MTHFR*, (Figure A.3) encoding a metabolic mediator that participates in the conversion of homocysteine to methionine. Given that homocysteine derivatives can activate NMDA receptors, there is a connection between *MTHFR* and glutamatergic signaling. Neurotransmission is furthermore the best represented pathway in migraine (Figure A.4). Nevertheless, migraine etiology, which continues to mystify basic and clinical researchers alike, is well represented by the proportional distribution of the other functional pathways in Figure A.4. The large variety of genetic mediators of migraine is consistent with the observed heterogeneous nature of this disorder.

A.3.2 Musculoskeletal conditions

Musculoskeletal pain conditions include temporomandibular disorder, low back pain, fibromyalgia, and chronic widespread pain. These disorders, whose heritability estimate is up to 50 %, have been recently characterized in terms of their phenotypic and genetic markers in (140). Additionally, a number of publications have reported a possible role for genetic variants in susceptibility to chronic musculoskeletal pain following a psychologically-traumatizing event, or stress-induced chronic pain.

Temporomandibular Disorder

The temporomandibular disorder (TMD) is the most frequently occurring class of orofacial pain conditions, with an estimated prevalence between 3 % and 12 % worldwide (141). This heterogeneous set of conditions exhibits great interindividual variability in manifestation and response to treatment as well as high comorbidity with other pain conditions. Their etiology, however, is still unclear. There is a lack of understanding of orofacial pain mechanisms and their modifying factors (142), which leads to a lack of accurate diagnoses and effective treatment. Given a reported heritability of 27 % (143), TMD genetics, reviewed recently in (144), has emerged as a powerful tool to aid in research and help clinicians deepen their comprehension of causal factors with the ultimate goal of providing effective and suitable treatment to patients.

While most studies to date have been done on small populations, the OPPERA (Orofacial Pain: Prospective Evaluation and Risk Assessment) project represents a multi-center, high-powered effort to identify genetic markers contributing to TMD (145). Although the OPPERA GWAS is underway (146), to date only targeted genotyping studies have been published by this group on a subset of this cohort and by others. These studies have nevertheless identified a number of genetic variants that implicate catecholaminergic, estrogenic, and serotonergic systems. Evidence also exists for the involvement of cytokines and other molecules in causal pain pathways, but their role is still poorly understood.

Involvement of the catecholaminergic system in TMD is exemplified by *COMT*, the most studied and cited gene in human pain genetics (147). This gene encodes the catechol-*O*-methyltransferase enzyme, which regulates the levels of catechol neurotransmitters. Its hypoactivity leads to a higher level of epinephrine, which potentiates beta adrenergic receptor-mediated pain signaling (148). *COMT* is a highly polymorphic gene with multiple functional SNPs. The most studied SNP is the non-synonymous *val158met* variation implicated in multiple pain conditions, mood disorders and cognitive function in a number of studies (149–154). Furthermore,

three major functional haplotypes in the coding region of the gene have shown strong association with response to noxious stimuli and risk of myogenous TMD in humans (155). A recently identified regulatory SNP in the 3'-untranslated region of *COMT* has led to the discovery of a functional alternate isoform of the COMT enzyme, shown *in vitro* to have preferred enzymatic activity toward dopamine, which may contribute to pain by lowering the level of dopamine rather than increasing the level of epinephrine (156). On the other hand, variants in *COMT* have been reported not to be significantly associated with TMD in a Turkish population (157).

Adrenergic receptor involvement has also been reported. Both hyperactive and hypoactive variants of beta adrenergic receptor *ADRB2* have been shown as possible modulators of TMD risk (158). Two alpha adrenergic receptors, *ADRA2C* and *ADRA1D*, have been reported as possibly significant TMD risk modulators (151).

Two common genetic variants in the human estrogen receptor *ESR1* have been implicated in painful temporomandibular joint (TMJ) disc displacement in a Brazilian female population (159). One of the associations has been replicated in Korean patients with TMJ osteoarthritis (TMJ-OA) (160), although another Korean cohort with TMD has shown a trend but no statistically significant association for the same SNP (161). Recently, another study has shown these variants as associated with the TMJ degenerative process, possibly via modulation of ESR1 activity in the bone (162). Given that TMJ is a well-documented target tissue for estrogen (163–165), it is not surprising to find estrogen receptor genetic variants involved in the pathophysiology of its disorders.

The serotonergic system has been implicated in TMD pathophysiology via polymorphisms in serotonin receptor, *HTR2A*, (151; 166–168) (not replicated in (169)), and serotonin transporter, *SLC6A4*, which have shown association in different populations (169; 170). *SLC6A4* has a 44bp insertion/deletion polymorphism within the promoter region, presenting two allelic forms, long (l) and short (s). It also has a polymorphic region of a 17bp-variable number of tandem repeats (VNTR) in its second intron. Both polymorphic regions have shown association with TMD: the promoter region in a Japanese population (169) and VNTR in a Turkish population (which has not shown an association for the promoter region) (170).

Numerous studies have shown that levels of proinflammatory cytokines are heightened in the temporomandibular joint fluid of TMD patients (171; 172) and that these levels correlate with greater pain sensitivity (173–176), perceived stress (177) and depression (178), all of which are phenotypes associated with TMD (179–182). In addition, elevated levels of proinflammatory cytokines have been consistently reported in circulating blood of patients with widespread pain (176; 183). Genetic

evidence for the involvement of cytokines in TMD has been therefore explored and reported (184). While no individual SNPs have shown association with TMD, two SNPs within the interleukin 8 gene, *CXCL8*, interacted similarly and significantly with a transforming growth factor *TGFB1* SNP, producing the greatest effect on TMD with widespread pain. A variant in interleukin 10, *IL10*, has also shown suggestive association (151).

Several loci have shown association as part of the OPPERA case-control study. DNA from chronic TMD patients was screened using a SNP panel of 358 genes implicated in pain through modulation of nociception, psychological state or inflammatory response. Of the top nine SNPs showing nominal statistically significant association, three are in the glucocorticoid receptor, *NR3C1*; one in serotonin receptor 2A, *HTR2A* (discussed above); one in muscarinic cholinergic receptor 2, *CHRM2*; two in calcium/calmodulin-dependent protein kinase 4, *CAMK4*; one in the interferon-related developmental regulator 1, *IFRD1*; and one in G protein-coupled receptor kinase 5, *GRK5* (151). Another study, designed under the hypothesis that TMD is a multifactorial syndrome related to a critical period of human life with a genetic and epigenetic basis, has investigated the possible involvement of 14 genes related to the folate cycle in TMD (185). Three genes encoding enzymes in the folate metabolizing pathway – serine hydroxymethyltransferase 1, *SHMT1*; methylenetetrahydrofolate dehydrogenase, *MTHFD1*; and methionine synthase reductase, *MTRR* – have shown significant associations with TMD. Their risk alleles were associated with odds ratios of having TMD that ranged from 2.35 to 3.99. In addition, the authors have reported that a gene-deletion polymorphism in *GSTM1*, encoding μ glutathione-S-transferase (which is associated with inflammatory oxidative stress), and the dopamine D4 receptor, *DRD4*, long allele of 48bp-repeat were associated with increased risk of TMD.

A recent study has investigated the association of SNPs occurring in five genes known to be important regulators of the TGF-beta signaling pathway, which plays a well-established role in the control of chondrocyte differentiation, matrix synthesis and homeostasis, and TMJ-OA. Of these, growth differentiation factor 5, *GDF5*; SMAD family member 3, *SMAD3*; and runt-related transcription factor 2, *RUNX2* were associated with the pathogenesis of TMJ-OA (186). GDF5 is known to play a key role in bone and cartilage morphogenesis as well as joint formation, and the same genetic variant has been previously associated with knee osteoarthritis (OA) in Europeans and Asians (187; 188). SMAD3 is a transcriptional modulator activated by TGF-beta that is crucial to the integrity of articular cartilage, and genetic variants in this gene have been implicated in pathogenesis of knee OA in European

and northeastern Chinese populations (189; 190). Lastly, RUNX2 is essential for osteoblast differentiation and skeletal morphogenesis. Its contribution to OA has been suggested in a large GWAS in Europeans (191).

In light of all the reported genetic associations, the suggested pathophysiology of TMD pain is modulated by pain regulators, inflammation-mediating cytokines, and relevant tissue morphogenesis proteins.

Low back pain

Low back pain, whose heritability is estimated to be up to 46 % (192) is mainly caused by disc disease (193) and characterized by the desiccation of the spinal disc matrix (194). Perhaps unsurprisingly, it has been associated with variants of genes encoding apoptosis-mediating caspase, *CASP9* (194; 195), and extracellular protein digestion enzyme, matrix metalloproteinase, *MMP1* (196; 197). Upstream of pathological proteoglycan degradation is the overactive mixture of proinflammatory cytokines, namely associated interleukins: interleukin 1A, *IL1A* (198–200) (which is also associated with treatment response (199)); interleukin 1 receptor antagonist, *IL1RN* (198; 201); and interleukin 18 receptor subunits encoded by *IL18R1* and *IL18RAP*, both of which modulate response to low back pain treatment (199). Other associated genes include growth differentiation factor 5, *GDF5*, which participates in skeletal tissue differentiation (195); beta-2 adrenergic receptor, *ADRB2*, which is associated with low back pain comorbid with neck pain (202); catechol-*O*-methyltransferase, *COMT* (203–206); estrogen receptor 1, *ESR1* (207); guanosine triphosphate cyclohydrolase, *GCH1* (208); and μopioid receptor, *OPRM1* (209) (although association of this gene with low back pain has not been replicated in (206). The results of genetic association studies outline a pathophysiology centered on disrupted tissue remodeling, with pain possibly resulting from an overabundance of proinflammatory signaling.

Fibromyalgia and chronic widespread pain

Fibromyalgia is diagnosed based on the presence of chronic widespread musculoskeletal pain with tenderness in at least 11 of 18 sites on the body, fatigue, and sleep disturbance, and it is frequently comorbid with mood disorders and other chronic pain disorders (210; 211). Given its heritability of approximately 50 % (212), genetic studies may provide valuable insight into the pathophysiology of fibromyalgia. Implicated genes show an over-representation of monoaminergic pathway members: dopamine D4 receptor, *DRD4* (213); catechol-*O*-methyltransferase, *COMT* (214–220); monoamine oxidase, *MAOA* (221); beta-2 adrenergic receptor, *ADRB2* (222); serotonin transporter, *SLC6A4* (223); serotonin receptor 2A, *HTR2A* (224–227);

GTP cyclohydrolase, *GCH1*, an enzyme critical to dopamine, serotonin, and nitric oxide production (228); and trace amine associated receptor, *TAAR1* (229), a G protein-coupled receptor with a regulatory role in dopaminergic neurotransmission (230). Other associations include genes encoding proteins more or less directly involved in neuronal inhibition and excitation, namely gamma-aminobutyric acid (GABA) A receptor, *GABRB3*, (229); sodium channel NaV1.7, *SCN9A* (231); and two genes identified in a fibromyalgia GWAS and awaiting replication: myelin transcription factor 1-like, *MYT1L*, which has a role in neuronal differentiation, and neurexin, *NRXN3*, a synaptic scaffolding stabilizer involved in glutamatergic and GABAergic neurotransmission (17). Additionally, associations for fibromyalgia have been reported for apolipoprotein, *APOE* (232), and guanylate binding protein, *GBP1* (229), a possible contributor to inflammatory disease (233).

Chronic widespread pain (CWP), which substantially correlates with fibromyalgia, is defined as pain present in the axial skeleton and in two contralateral bodily quadrants for at least three months (234). A study targeting genes of the HPA axis in CWP has found an association for glucosteroid binding globulin, *SERPINA6*; corticotropin-releasing hormone-binding protein, *CRHBP*; pro-opiomelanocortin, *POMC*; and adrenocorticotropic hormone receptor, *MC2R* (226). A subsequent GWAS meta-analysis conducted by the same group has found a locus of susceptibility to chronic widespread pain in a non-conding region between the chaperonin-containing T-complex polypeptide 1 (TCP1)-complex-5 gene, *CCT5*, and FAMily with sequence similarity 183 member B gene, *FAM183B* (18). While the function of the latter protein is not known, CCT5 is a structural scaffolding subunit that interacts with protein phosphatase PP4C and may be involved in central sensitization (18). Together, the genetic landscapes of fibromyalgia and chronic widespread pain implicate a rewired nociceptive signaling system, possibly disrupted by excessive stress response.

Stress-induced chronic pain

Recently, a number of studies have shown an overlap of symptoms between post-traumatic stress disorder and musculoskeletal pain conditions (235; 236). Associations with glucocorticoid receptor co-chaperone, *FKBP5* (237), and corticotropin releasing hormone-binding protein, *CRHBP* (238), two HPA axis-related genes, as well as mu opioid receptor, *OPRM1*, and chronic musculoskeletal pain following a motor vehicle collision have been reported in a cohort of 950 Caucasian Americans (239) with replication in a sexual assault survivors' cohort for *FKBP5* (237). The same study has also shown that catechol-*O*-methyltransferase, *COMT*, is associated with musculoskeletal pain several weeks following motor vehicle collision (150; 240).

Ballina *et al.* have furthermore reported a correlation between the same *OPRM1* variant, A118G, and muscle pain after sexual assault (241). They have also shown that widespread pain after a motor vehicle collision is associated with pre-accident depression, suggesting that pain was a factor of the patient's psychological characteristics (presumably a proxy for genetic predisposition) rather than tissue injury (242). In sum, these findings suggest that genetic variability in the endogenous stress-management and analgesia systems modulate susceptibility to musculoskeletal pain conditions triggered by stress and psychological trauma.

Musculoskeletal condition genetics: summary

The above overview of genes associated with musculoskeletal chronic pain conditions highlights a possible underlying pathophysiology. By the number of reports, the contribution from the catecholaminergic system, represented by *COMT* and *ADRB2* associations, is seconded by the involvement of the serotonergic system, exemplified by associations with *HTR2A* and *SLC6A4* (Figure A.5). In terms of functional pathways, neurotransmission is the most common contributor (Figure A.6), followed by immune response mediators. While neurotransmitters and inflammatory cytokines are directly involved in pain signaling, the other three functional groups may contribute to musculoskeletal conditions by increasing susceptibility to the underlying tissue damage.

A.3.3 Neuropathic pain disorders

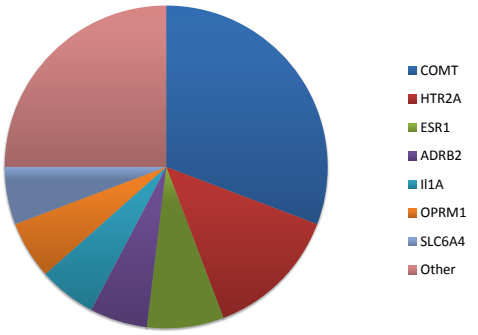
Neuropathic pain disorders result from nerve dysfunction. The effect of genetic contributors differs according to the origin of this dysfunction. In rare monogenic disorders, somatosensory function is maligned by causal mutations in a single gene. The more common disorders with a chronic neuropathic component are cancer, diabetes, postoperative pain, and trigeminal neuralgia. For this class of conditions, genetic variants have been shown to modify an individual's susceptibility to neuropathic pain on the background of a different underlying cause.

Rare neuropathic pain disorders

Rare neuropathic pain conditions fall into two categories: painless disorders and painful disorders. Congenital insensitivity to pain with anhidrosis, or inability to sense and respond to noxious stimuli and to sweat, was the first monogenic neuropathic disorder to be reported. The causal mutations were identified in the *NTRK1* gene, which encodes neurotrophic tyrosine kinase receptor (243), an extensively replicated finding (244–256). A related disorder, congenital insensitivity to pain, has been reported to be caused by loss-of-function mutations in sodium channel Nav1.7-encoding *SCN9A* (4; 257–262). Interestingly, gain-of-function mutations in

Gene	# of citations
COMT	16
HTR2A	7
ESR1	4
ADRB2	3
IL1A	3
OPRM1	3
SLC6A4	3
Other	13

(a)

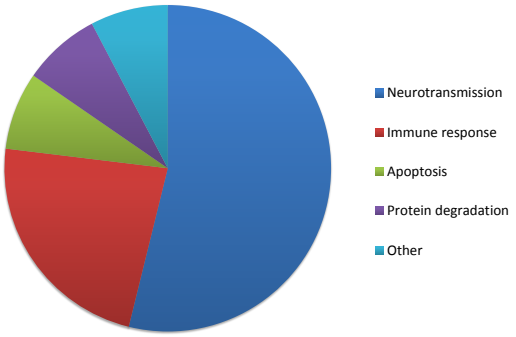


(b)

Figure A.5: **Genetic loci associated with musculoskeletal pain disorders, quantified by the number of genetic association studies.** Only genes reported to be associated with a musculoskeletal pain disorder in at least two studies are included, and only genes with association reported in at least three studies are listed individually. Genes with fewer reported associations are grouped under "Other." Abbreviations: *COMT*, catechol-O-methyl transferase; *HTR2A*, 5-hydroxytryptamine (serotonin) receptor 2A; *ESR1*, estrogen receptor 1; *ADRB2*, beta-2 adrenergic receptor; *GCH1*, GTP cyclohydrolase; *OPRM1*, mu-1 opioid receptor; *SLC6A4*, solute carrier family 6 (serotonin transporter).

Condition	# of citations
Neurotransmission	7
Immune response	3
Apoptosis	1
Protein degradation	1
Other	1

(a)



(b)

Figure A.6: **Functional pathways of genetic loci associated with musculoskeletal disorders quantified by the number of loci.** Only genes with at least one replicated association are included. For genes reported to be involved in multiple pathways, the pathway of the translated gene's most direct involvement was chosen.

another sodium channel, Nav1.9, *SCN11A*, have also been implicated in this condition (5; 263). Hyperactivity in Nav1.9 prolongs cell membrane depolarization thereby inactivating Nav1.7 and Nav1.8 – the drivers of the action potential in nociceptive neurons – and blocking pain signal transmission.

Painful rare neuropathic pain disorders include erythromelalgia, characterized by painful swelling and severe redness in feet and hands, and paroxysmal extreme pain disorder, characterized by rectal, periocular and perimandibular pain. Causal *SCN9A* mutations in erythromelalgia, discovered (7) and replicated in a number of Chinese Asian families (264–268) and Caucasian families (269–273) have been shown to change the electrophysiological properties of dorsal root ganglion (DRG) neurons, believed to affect pain sensitivity (269; 274; 275). The effects of paroxysmal extreme pain disorder causal mutations (276–278) have been postulated to lie on a physiological continuum with erythromelalgia. Estacion *et al.* have demonstrated that alleles responsible for the former condition contribute to impaired fast inactivation in pain-transmitting neurons, while those responsible for the latter condition contribute to a lower firing threshold, slow deactivation and enhanced ramp currents (277).

Cancer pain

Unlike the rare conditions discussed above, in which chronic pain is caused by genetic mutations of large effect in one gene locus, genetic variants contributing to cancer pain are found in several loci and their effect on pain is much more moderate. The cause of cancer pain is usually a growing tumor directly stimulating nociceptors, nerve damage during chemotherapy (279), and/or inflammatory cytokines released by cancerous cells (280). Interleukin-8, *CXCL8* (280); interleukin 1 receptor, *IL1R1*; and interleukin 13, *IL13* (281), have been reported to be associated with cancer pain severity. Reyes-Gibby *et al.* have reported on the effect of inflammation-related gene polymorphisms on lung cancer pain severity, with associations for prostaglandin-endoperoxide synthase 2, *PTGS2*; tumor necrosis factor, *TNF*; and NF-kappa B inhibitor alpha, *NFKBIA*, with severe pain (282). The additive effect of variants in *NOS3*, *IL1B*, *TNFRSF1B*, *PTGS2*, and *IL10RB* has been reported to be implicated in the severe symptom cluster in cancer patients, characterized by high pain intensity, depression, and fatigue (283). As with diabetes, while it may not be directly involved in the pathophysiology of the underlying condition, hypersensitivity of the body's defense system appears to be conducive to more pain accompanying cancer.

Diabetic neuropathy

Diabetic neuropathy, characterized by pain at one extreme and insensitivity at the other is observed in up to 50 % of diabetic patients (284). Exacerbated by prolonged diabetic condition, glycemic mismanagement (285), and disruption of nerve microvasculature (284) this neuropathy presents as a multifactorial pathology that is likely to be substantially driven by genetic factors. Unfortunately, diabetic pain genetic association studies are scarce, and existing results have to date painted a far-from-complete picture of its pathophysiology. Nevertheless, it is noteworthy that a hypofunctional polymorphism in mu opioid receptor, *OPRM1*, is associated with foot ulcer pain in diabetic patients (286), while hyperfunctional variants in purinergic receptor 7, *P2RX7*, are reported to be associated with higher pain sensitivity in diabetic neuropathic women (287). Insufficient endogenous pain regulation (*OPRM1*) and excess high alert signaling (*P2RX7*) appear to contribute to baseline nociceptive hypersensitivity, which exacerbates diabetes-related pain.

Postoperative pain

Postoperative pain is usually considered persistent, or chronic, when it is reported at least three months after surgery. The complementary nociceptive and inhibitory systems are implicated in several examples from postoperative pain studies: Kim *et al.* have reported an association between a variant in GTP hydrolase, *GCH1*, and postoperative pain (288); Tegeder *et al.* have identified an association between postoperative pain and a haplotype of the same gene (208) (not replicated either at the SNP or haplotype level in (289)); and two groups have reported that mu opioid receptor, *OPRM1*, genotypes predict the extent of chronic postoperative pain (290; 291). The latter group has shown that the effect of the *OPRM1* variant is sex-specific, such that allele G118 confers risk of pain in women but correlates with less pain in men. Similarly, a variant in voltage-gated potassium channel subunit, *KCNS1*, has been reported as correlated with postoperative chronic pain intensity in two limb amputation cohorts and one post-mastectomy cohort (292). Another post-mastectomy cohort study (293) has reported an association for a purinergic receptor 7, *P2RX7*, variant. Additionally, a *COMT* polymorphism has been reported to be associated with pain one year after lumbar discectomy (203).

Genetic variants of inflammatory cytokines have also been demonstrated to play a role in the risk of neuropathic pain following surgery. Stephens *et al.* have reported an association between a SNP of interleukin 1 receptor, *IL1R2*, and a haplotype within interleukin 10, *IL10*, with persistent breast pain after surgery for breast cancer (294).

Trigeminal neuralgia

Trigeminal neuralgia is the most common type of neuralgia in adults with an estimated annual incidence of 4 to 13 per 100 000 people (295; 296). Trigeminal neuralgia is characterized by sudden, usually unilateral, severe, brief, stabbing recurrent episodes of pain within the distribution of one or more branches of the trigeminal nerve, triggered by innocuous stimuli (297). To date, the precise mechanisms of trigeminal neuralgia remain unclear, and only a few studies have been conducted on the genetics of this condition.

The role of the serotonin transporter, *SLC6A4*, 44bp insertion/deletion polymorphism in susceptibility to trigeminal neuralgia and its clinical features, especially the pain severity and treatment response to analgesics, has been recently investigated (298). The *s* variant carriers had a significantly higher risk of trigeminal neuralgia pain severity and of carbamazepine treatment failure. These findings are contrary to those reported for TMD (169); however, chronic pain conditions of musculoskeletal and neuropathic origins have substantially different components, and this apparent contradiction is not surprising.

Other painful peripheral neuropathic conditions

Several other peripheral neuropathic pain conditions have been reported. Haplotypes in voltage-gated potassium channel subunit, *KCNS1*, have shown association with pain intensity in HIV-associated sensory neuropathy among black South Africans (289). Transient receptor potential channels, *TRPA1* and *TRPV1*, have been reported to affect somatosensory sensitivity in neuropathic pain patients who suffered from a variety of neuropathic conditions (299). *SCN9A* has likewise been reported to be associated with unexplained chronic neuropathic pain (300).

Three sodium channels have also been reported to be associated with a more common neuropathic pain condition, painful small fiber neuropathy. Affecting small-diameter A-delta and C fibers and characterized by sudden bouts of pain originating in the extremities, this condition has been reported to be associated with Nav1.7, *SCN9A* (301; 302); Nav1.8, *SCN10A* (303–305); and Nav1.9, *SCN11A* (306; 307). The relevant mutations in these channels have been functionally characterized and found to contribute to sensory neuron hyperexcitability (301–304; 306; 307).

Neuropathic condition genetics: summary

Unlike in migraine and musculoskeletal pain conditions, our understanding of genetic contributors to neuropathic pain is largely derived from rare familial mutations and is dominated by sodium channels. While Nav1.7, and to a lesser extent Nav1.8 and Nav1.9, have been repeatedly reported as causal in rare neuropathic pain conditions,

the direction and extent of functional effect on the disease phenotype is tied to the specific mutation or mutations within each locus. Moreover, extensive pedigree segregation analysis and electrophysiological characterization has occasionally shown previously reported causal mutations as benign or conferring a mild risk (4; 308).

Genetic variants underlying common neuropathic pain conditions also point to modulation of neurotransmission and related pathways. Three of the four genes whose association has been replicated at least once – *GCH1*, a regulator in the dopamine, serotonin, and nitric oxide biosynthesis pathway; *KCNS1*, a voltage-gated potassium channel subunit; and *OPRM1*, one of the body's endogenous pain regulators – implicate dysregulation in pain processing. The other gene, twice reported to be associated with cancer pain, is *PTGS2*, which indicates involvement of the immune system.

A.3.4 Visceral pain disorders

Visceral pain disorders, reviewed in (300; 309), are characterized by pain stemming from visceral organs. At least in part due to the lack of consensus on diagnostic criteria for these conditions, genetic studies have been few and have provided but a glimpse into their pathophysiology, with tenuous connections between implicated genes. Possible involvement of genetic polymorphisms has been reported for chronic pelvic pain, chronic pancreatitis and interstitial cystitis.

Chronic pelvic pain is a pathology believed to result from certain bacterial infections and sexually-transmitted disease sequelae (310). Evidence for inflammatory dysregulation comes from a study showing that chronic pelvic pain patients were more likely to express the genotype associated with reduced production of the regulatory cytokine interleukin 10, *IL10* (311). An early study by Riley *et al.* has implicated a short tandem repeat region of the X chromosome near the phosphoglycerate kinase gene, *PGK1*, in chronic prostatitis/chronic pelvic pain (312). Two other genes possibly implicated in chronic pelvic pain are manganese superoxide dismutase, *MNSOD* (313), and androgen receptor, *AR* (310).

Several genes have been reported associated with chronic pancreatitis with pain. Variants of cystic fibrosis transmembrane conductance regulator, *CFTR* (314–316) (a chloride channel whose more damaging mutations lead to cystic fibrosis); serine protease inhibitor, *SPINK1* (317; 318), which regulates trypsinogen; cationic trypsinogen, *PRSS1* (319), which in its hyperactive form essentially autodigests the pancreas; and trypsin inhibitor, *PSTI* (316), have all been reported to be associated with chronic pancreatitis with pain. *PRSS1* also carries mutations that cause hereditary pancreatitis (316; 320–323), as does *SPINK1* (324).

Interstitial cystitis, characterized by bladder pain, has been found associated with a potentiating variant of sodium channel NaV1.7, *SCN9A* (325).

Visceral condition genetics: summary

Genetic studies of visceral conditions have thus far not painted a clear picture of the biological pathways involved in their pathophysiology. *SCN9A*, whose involvement in visceral chronic pain has been reported four times, appears to be the most frequent contributor to chronic pain accompanying visceral disorders. Given this gene's well-established role in a number of rare monogenic pain conditions, its possible role in visceral pain may be yet another manifestation of disrupted pain signaling. Furthermore, the four genes whose associations with visceral disorders have been replicated at least once represent three different functional pathways: *SCN9A* – neurotransmission, *PRSS1* and *SPINK1* – protein degradation, and *CFTR* – immune response. This evidence from genetic studies to date suggests that visceral pain disorders may not have a common pathophysiology and are rather grouped together primarily based on the anatomical proximity of the affected organs.

A.4 Conclusion

Genetic studies conducted in the last two decades have been invaluable in elucidating the molecular pathophysiology mechanisms of chronic pain conditions. While these studies have occasionally suffered from contradictory results or insufficient statistical power to confirm the involvement of specific genes, the network of causal mechanisms is nevertheless gradually coming into focus. Genetic variants contributing to chronic pain conditions characterize these conditions as multifactorial pathologies with overlapping etiologies. All chronic pain condition categories show an enrichment for genes involved in neurotransmission, underscoring the importance of neuronal signaling – specifically ascending nociceptive and descending inhibitory signaling – in pain chronicity. Nevertheless, secondary functional pathways differ between the etiologically grouped categories and shed light on possible pathophysiological differences between them. This suggests that distinct combinations of genetic variants, presumably interacting with environmental factors, determine the specific pathology that develops. Thus identification of genetic contributors of chronic pain conditions builds our understanding not only of the genotypic structure of these diseases but also of their molecular pathophysiology.

A.4 Conclusion

Furthermore, currently available chronic pain treatments are fraught with significant obstacles, including unmanageable side effects (326; 327) and variable effectiveness at the population level (328–330), which are attributable to interindividual variability in pharmacokinetic and pharmacodynamic properties of analgesics. Perhaps even more importantly, given that chronic pain conditions are a heterogeneous class of disorders, driven by different pathways of vulnerability that include differential molecular genetic contribution, genetic studies promise to identify key molecular markers of susceptibility and targets for personalized treatment of chronic pain. Therefore the results of genetic studies should be exploited for drug development targeting of molecular pathways unique – or as close to unique as possible – to each pathology.

Table A.1: Genes reported in genetic association studies of chronic pain conditions. Abbreviations: FHM, familial hemiplegic migraine; TMD, temporomandibular disorder.

Gene	Function/Pathway	Condition(s)	Citation
ACAN	Structural protein	Low back pain	(331)
ACE	Other	Migraine	(43; 54; 59–63; 332–334)
ADAMTSL4	Protein degradation	Migraine	(16)
ADARB2	Neurotransmission	Migraine	(13)
ADRA1D	Neurotransmission	TMD	(151)
ADRA2C	Neurotransmission	TMD	(151)
ADRB2	Neurotransmission	TMD	(158)
		Low back pain	(202)
		Fibromyalgia	(222)
AJAP1	Other	Migraine	(15; 16; 66; 111)
ANKK1	Other	Migraine	(100)
APOA1BP	Metabolism	Migraine	(15)
APOE	Metabolism	Fibromyalgia	(232)
AR	Other	Visceral	(310)
ARMS2	Other	Migraine	(16)
ASTN2	Other	Migraine	(12; 16; 66)
ATP1A2	Neurotransmission	FHM2	(35)
ATP5B	Other	Migraine	(15)
BDNF	Cellular growth	Migraine	(115–117)
C7orf10	Metabolism	Migraine	(15; 16; 66; 111)
CACNA1A	Neurotransmission	FHM1	(34)
CAMK4	Other	TMD	(151)
CARF	Other	Migraine	(16)
CASP9	Apoptosis	Low back pain	(194; 195)
CCM2L	Other	Migraine	(16)
CCR2	Immune response	Migraine	(58)
CCT5	Structural protein	Fibromyalgia	(18)
CFDP1	Other	Migraine	(16)
CFTR	Immune response	Visceral	(314–316)
CGRP	Neurotransmission	Migraine	(117)
CHRM2	Neurotransmission	TMD	(151)
COMT	Neurotransmission	Fibromyalgia	(214–220)
		Low back pain	(203–206)
		Postoperative pain	(203)
		Migraine	(104)
		Stress-induced chronic pain	(150; 240)
		TMD	(151; 152; 155–157)
CPQ	Neurotransmission	Migraine	(10)
CRHBP	Neurotransmission	Stress-induced chronic pain	(238)
		Fibromyalgia	(226)
CXCL8	Immune response	TMD	(184)
		Cancer pain	(280)
DAO	Neurotransmission	Migraine	(118)
DBH	Neurotransmission	Migraine	(97–100)

DOCK4	Other	Migraine	(16)
DRD2	Neurotransmission	Migraine	(93; 94; 100)
DRD4	Neurotransmission	Migraine	(94; 101; 102)
		TMD	(185)
		Fibromyalgia	(213)
EDNRA	Other	Migraine	(40; 41)
ESR1	Other	TMD	(159–162)
		Migraine	(122–127; 335)
		Low back pain	(207)
ESR2	Other	Migraine	(124; 125)
FAM183B	Other	Fibromyalgia	(18)
FGF6	Immune response	Migraine	(16)
FHL5	Other	Migraine	(15; 16; 66; 111)
FKBP5	Immune response	Stress-induced chronic pain	(237)
FSHR	Other	Migraine	(124)
FUT9	Metabolism	Migraine	(15)
GABRB3	Neurotransmission	Migraine	(105; 106)
		Fibromyalgia	(107; 229)
GBP1	Immune response	Fibromyalgia	(229)
GCH1	Metabolism	Fibromyalgia	(228)
		Peripheral neuropathy	(289)
		Postoperative pain	(208; 288)
GDF5	Cellular growth	Low back pain	(195)
		TMD	(186)
GJA1	Other	Migraine	(16)
GPR149	Other	Migraine	(16)
GRIA1	Neurotransmission	Migraine	(82–84)
GRIA3	Neurotransmission	Migraine	(82; 84)
GRK5	Other	TMD	(151)
GSTM1	Metabolism	TMD	(185)
HCRTR1	Other	Migraine	(109)
HEY2	Other	Migraine	(16)
HLA-DRB1	Immune response	Migraine	(131)
HPSE2	Metabolism	Migraine	(16)
HTR2A	Neurotransmission	TMD	(151; 166–169)
		Fibromyalgia	(224–227)
HTR7	Neurotransmission	Migraine	(13)
IFRD1	Immune response	TMD	(151)
IGSF9B	Neurotransmission	Migraine	(16)
IL10	Immune response	Postoperative pain	(294)
		TMD	(151)
		Visceral	(311)
IL10RB	Immune response	Cancer pain	(283)
IL13	Immune response	Cancer pain	(281)
IL18R1	Immune response	Low back pain	(199)
IL18RAP	Immune response	Low back pain	(199)
IL1A	Immune response	Low back pain	(199; 200)
IL1B	Immune response	Migraine	(71)
		Cancer pain	(283)
IL1R1	Immune response	Cancer pain	(281)
IL1R2	Immune response	Postoperative pain	(294)
IL1RN	Immune response	Low back pain	(201)
IL9	Immune response	Migraine	(58)
INSR	Metabolism	Migraine	(132)
ITPK1	Metabolism	Migraine	(16)
JAG1	Metabolism	Migraine	(16)
KCNAB3	Neurotransmission	Migraine	(113)
KCNG4	Neurotransmission	Migraine	(113)
KCNK18	Neurotransmission	Migraine	(6; 112)
KCNS1	Neurotransmission	Sensory neuropathy	(289)
		Postoperative pain	(289; 292)
LDLR	Metabolism	Migraine	(129; 130)
LRP1	Neurotransmission	Migraine	(11; 16; 66; 67)
LRRIQ3	Other	Migraine	(16)
LTA	Immune response	Migraine	(70; 74–76)
MAOA	Neurotransmission	Fibromyalgia	(221)
		TMD	(336)
MC2R	Neurotransmission	Fibromyalgia	(226)
MED14	Metabolism	Migraine	(16)
MEF2D	Apoptosis	Migraine	(12; 16; 66)
MMP1	Protein degradation	Low back pain	(196; 197)
MMP2	Protein degradation	Low back pain	(337)
MMP3	Protein degradation	Low back pain	(338)
MMP16	Protein degradation	Migraine	(15; 16; 66)
MNSOD	Other	Visceral	(313)
MPPED2	Other	Migraine	(16)

MRVI1	Other	Migraine	(16)
MTDH	Other	Migraine	(10; 12; 16)
MTHFD1	Other	TMD	(185)
MTHFR	Metabolism	Migraine	(42–56; 63; 335; 339; 340)
MTRR	Other	TMD	(185)
MYT1L	Other	Fibromyalgia	(17)
NFKBIA	Immune response	Cancer pain	(282)
NGFR	Cellular growth	Migraine	(14)
NOS3	Neurotransmission	Migraine	(57; 58)
		Cancer pain	(283)
NOTCH3	Other	Migraine	(64; 65)
NOTCH4	Other	Migraine	(16; 136)
NR3C1	Immune response	TMD	(151)
NRP1	Apoptosis	Migraine	(16)
NRXN3	Neurotransmission	Fibromyalgia	(17)
NTRK1	Other	Congenital insensitivity to pain	(244–256)
OPRM1	Neurotransmission	Stress-induced chronic pain	(239; 241)
		Low back pain	(206; 209)
		Postoperative pain	(290; 291)
		Diabethic neuropathic pain	(286)
P2RX7	Neurotransmission	Postoperative pain	(293)
		Diabethic neuropathic pain	(287)
PGK1	Metabolism	Visceral	(312)
PGR	Other	Migraine	(128)
PHACTR1	Metabolism	Migraine	(12; 16; 66)
PLCE1	Other	Migraine	(16)
POMC	Neurotransmission	Fibromyalgia	(226)
PRDM16	Other	Migraine	(11; 16; 66; 67; 133; 134)
PRRT2	Other	FHM	(37; 38)
PRSS1	Protein degradation	Visceral	(316; 319–323)
PTGS2	Immune response	Migraine	(78; 79)
		Cancer pain	(282; 283)
RAMP1	Other	Migraine	(119; 120)
REST	Other	Migraine	(16)
RNF213	Other	Migraine	(16)
RUNX2	Other	TMD	(186)
SCN1A	Neurotransmission	FHM3	(33; 36)
SCN9A	Neurotransmission	Erythromelalgia	(7; 264–275; 341)
		Paroxysmal estreme pain disorder	(276–278)
		Congenital insensitivity to pain	(4; 257–262)
		Peripheral neuropathy	(300–302)
		Fibromyalgia	(231)
		Visceral	(325)
SCN10A	Neurotransmission	Peripheral neuropathy	(303–305)
SCN11A	Neurotransmission	Congenital insensitivity to pain	(5; 263)
		Peripheral neuropathy	(306; 307)
SERPINA6	Immune response	Fibromyalgia	(226)
SHMT1	Metabolism	TMD	(185)
SLC24A3	Neurotransmission	Migraine	(16)
SLC6A4	Neurotransmission	Fibromyalgia	(223)
		Migraine	(86–89)
		TMD	(169; 170)
		Trigeminal neuralgia	(298)
SMAD3	Other	TMD	(186)
SPINK1	Protein degradation	Visceral	(317; 318; 324)
STAT6	Other	Migraine	(15)
TAAR1	Neurotransmission	Fibromyalgia	(229)
TBC1D7	Cellular growth	Migraine	(15)
TGFB1	Cellular growth	TMD	(184)
		Migraine	(58)
TGFBR2	Cellular growth	Migraine	(12; 16; 66)
TNF	Immune response	Migraine	(58; 69–75)
		Cancer pain	(282)
TNFRSF1B	Immune response	Migraine	(77)
		Cancer pain	(283)
TRPA1	Neurotransmission	Sensory neuropathy	(299)
TRPM8	Neurotransmission	Migraine	(11; 12; 16; 66)
TRPV1	Neurotransmission	Migraine	(139)
		sensory neuropathy	(299)
TRPV3	Neurotransmission	Migraine	(139)
TSPAN2	Other	Migraine	(15; 16; 66)
WSCD1	Metabolism	Migraine	(16)
YAP1	Apoptosis	Migraine	(16)
ZCCHC14	Other	Migraine	(16)

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B

Appendix B: Genetic studies of human neuropathic pain conditions: a review

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Numerous studies have shown associations between genetic variants and neuropathic pain disorders. Rare monogenic disorders are caused by mutations of substantial effect size in a single gene, whereas common disorders are likely to have a contribution from multiple genetic variants of mild effect size, representing different biological pathways. In this review, we survey the reported genetic contributors to neuropathic pain and submit them for validation in a 150,000-participant sample of the U.K. Biobank cohort. Successfully replicated association with a neuropathic pain construct for 2 variants in *IL10* underscores the importance of neuroimmune interactions, whereas genome-wide significant association with low back pain (P value = 1.3e-8) and false discovery rate 5% significant associations with hip, knee, and neck pain for variant rs7734804 upstream of the *MAT2B* gene provide evidence of shared contributing mechanisms to overlapping pain conditions at the molecular genetic level.

B.1 Introduction

Neuropathic pain arises from a lesion or disease of the somatosensory system (1). While some conditions have a known genetic cause, others develop as part of disease sequelae or post-traumatic complications.

The defining feature is an aberrant nociceptive network manifesting as pain occurring spontaneously or without adequate stimulation (2; 3). In the case of rare familial disorders, abnormal nociceptive signalling is genetically encoded, and many causal variants are known. Acquired neuropathic pain may develop secondarily to another condition, such as diabetes or cancer, in which nerve damage is often a consequence of disease progression. Alternatively, nerve damage or lesion may occur during physical trauma or surgery and result in a neuropathic pain condition. In all these cases, susceptibility to chronic pain varies beyond what environmental factors can explain. Although twin studies for common neuropathic pain conditions have not been done, substantial heritability has been reported in multiple other chronic pain conditions (4) – including back and neck pain, which often have a neuropathic component. Animal models of neuropathic pain have likewise revealed a significant genetic contribution (5; 6).

During the past decade the number of studies aiming to identify genetic factors in neuropathic pain conditions has grown in the hope of elucidating the molecular risk factors and identifying treatment targets. In this review, we summarise these studies and present the current landscape of neuropathic pain molecular pathophysiology as it has been informed by them.

B.2 Methods

To obtain a list of original studies reporting genetic association or linkage analysis with neuropathic pain conditions, we used the search method described in (7). Briefly, after drawing the initial list from the Human Pain Genetics Database (https://humanpaingenetics.org/hpgdb), a search was conducted in Google Scholar using the name of each disorder and one of the following terms: "genetic association", "variant", or "polymorphism". Additionally, we performed a search in PubMed, using the string: (((gene OR variant OR polymorphism) AND neuropath*) AND pain*) under the category "Text Word". Lastly, recent reviews of neuropathic pain genetics were perused for studies overlooked using the above methods. Studies

reporting association with a multi-symptom condition were included if neuropathic pain was one of the described symptoms, using the rationale that pleiotropic genetic loci should be considered neuropathic pain modulators whether or not they affect other clinical phenotypes. While publications were primarily screened by title and abstract, the text and relevant tables were perused if clarifications were necessary.

After compiling the list of genes containing variants with reported associations in common neuropathic pain conditions, we took all variants with a minimum minor allele frequency of 1% (except human leukocyte antigen, HLA-region variants due to their complex haplotypic structure) and checked them for validation in the U.K. Biobank, UKBB (Application no.20802). This public repository of genotypic and phenotypic data for 500,000 U.K. individuals, aged 40-69, is a dataset of unprecedented proportions in human genetic association studies (8). To construct a neuropathic pain phenotype, we grouped the following conditions (self-reported or determined by interview with a clinical nurse, UKBB Data-Field, hereafter DF, 20002): peripheral neuropathy (code 1255), diabetic neuropathy and ulcers (1468), shingles (1573), trigeminal neuralgia (1523), sciatica (1476), spinal stenosis (1536), peripheral nerve injury (1394), trapped or compressed nerve (1257), prolapsed or slipped disc (1312), varicella zoster virus (1674), and peripheral nerve disorder (1254). Individuals self-reporting one or more of these conditions were classified as cases and those reporting no pain conditions (DF 6159) as controls. In addition to neuropathic pain, we checked the list of variants in Table B.1 for association with four site-specific pain conditions (>3 months' duration) in the UKBB: back (DF 3571), hip (DF 3414), knee (DF 3773), and neck (DF 3404). Controls were the same as in the neuropathic pain group (no pain under DF 6159). To test for association with these phenotypes, we ran regression analyses on the available sample of 150,000 individuals of predominantly Caucasian ancestry using SNPTEST (9), version 2.5.2.

B.3 Results

The literature review findings are divided into rare monogenic disorders and common disorders with multiple associated risk loci and a complex etiology. The two classes of disorders are studied using different approaches: while rare conditions require linkage analysis of multi-generation pedigrees, common conditions require association studies of large cohorts of unrelated individuals. Section 3.1 is devoted to findings from familial rare-variant studies, and section 3.2 focuses on common

disorders with neuropathic pain as a secondary attribute.

B.3.1 Monogenic disorders

As the name suggests, monogenic disorders are often caused by variants in a single gene. The effect of such a variant can either exacerbate nociception or annul it. While not painful, the latter class of disorders is tied to genetic loci that directly participate in pain signalling or contribute to the vitality of sensory neurons and are therefore equally important to our understanding of pain processing. In fact, some of the same genes that harbour painful neuropathic variants also carry mutations leading to painless states.

Painful rare monogenic disorders

Erythromelalgia is marked by redness and painful swelling of hands and feet, symptoms that have been attributed to C-fibre hypersensitivity (10). Its hereditary form, primary erythromelalgia, has shown linkage to rare hyperfunctional variants in sodium channel NaV1.7 (*SCN9A*), discovered as causal (11) and repeatedly replicated in Chinese (12–16) and Caucasian (17–21) individuals.

The implicated *SCN9A* variants have been reported to change the electrophysiological properties of dorsal root ganglion (DRG) neurons, thereby affecting nociceptive signalling (17; 22; 23). The magnitude of effect on these functions appears to modulate the timing of disease onset. Thus, a variant with a smaller effect on hyperpolarization has been reported to be associated with later onset of erythromelalgia (24). An alternative theory posits that *SCN9A* variants affecting different electrophysiological properties translate to different neuropathic conditions (25). In cellular assays, this group has demonstrated that alleles responsible for erythromelalgia disrupted fast inactivation in nociceptors, while alleles that lower firing thresholds, slow deactivation, and potentiate currents result in paroxysmal extreme pain disorder (25). The latter is also a rare neuropathic disorder, which manifests as rectal, periocular, and perimandibular pain, and affected individuals have been reported to carry gain-of-function variants in *SCN9A* (25–27). The genetic contribution of *SCN9A* variants to paroxysmal extreme pain disorder and their cellular phenotype has been also reported in (28). This gene's variants have likewise been reported to be associated with unexplained chronic neuropathic pain (29).

Sodium channels NaV 1.7, NaV 1.8 and NaV 1.9 (*SCN9A*, *SCN10A* and *SCN11A*, respectively), have been found to harbour variants involved in a set of conditions collectively known as idiopathic painful small fibre neuropathies. These conditions affect small-diameter A-delta and C fibers, and their clinical manifestations include sudden bouts of pain propagating inward from the extremities. Associations with

SCN9A (29–31), *SCN10A* (32–34), and *SCN11A* (35–37) are supported by their cellular phenotype – hyperexcitability in dorsal root ganglion neurons (30–33; 35; 36).

Aside from sodium channels, variants in 4 other genes have been reported in connection with painful peripheral neuropathies. α-galactosidase A, *GLA*, has been reported in a small fibre neuropathy patient (38). Myelin protein zero, *MPZ*, has been reported in a family with debilitating neuropathic pain and demyelination. A variant in a subunit of kinesin, *KIF5A*, a protein involved in intracellular motility, has been reported to cause a form of hereditary spastic paraplegia with axonal neuropathy and pain (39). Individuals with a rare case of late-onset hereditary peripheral neuropathy have been reported to carry a variant in α-*N*-acetyl-glucosaminidase, *NAGLU*, (40).

Painless rare monogenic disorders

Among rare congenital sensory disorders, a class of conditions defined by insensitivity to pain has been linked to hypofunctional variants in *SCN9A* (41–47), and hyperfunctional variants in *SCN11A* (48; 49). These findings are consistent with the electrophysiological properties of the two sodium channels. Increased activity in NaV1.9 leads to longer neuronal depolarisation, which inhibits NaV1.7 and NaV1.8 activity in nociceptors, effectively shutting down pain signal transmission.

Additionally, insensitivity to pain is one of the defining symptoms in a group of disorders known collectively as hereditary sensory and autonomic neuropathies (HSANs). Primarily, variations in autonomic symptoms and genetic causes segregate these disorders into eight major subtypes, seven of which include insensitivity to pain. The different pathways leading to pain insensitivity are tagged by the 11 operative genes whose variants have been found in affected individuals: *SPTLC1*, *DMNT1*, *WNK1*, *KIF1A*, *RETREG1*, *SCN9A*, *ELP1*, *NTRK1*, *NGF*, *SCN11A*, and *PRDM12*. *SPTLC1* encodes a subunit of serine palmitoyltransferase, whose variant-compromised activity contributes to neuronal toxicity and death. *DNMT1* encodes a DNA methyltransferase, whose impaired function disrupts neuronal maintenance. Variants in these two genes have been reported as causal in HSAN, type I (50–56). *WNK1* encodes WNK lysine deficient protein kinase 1 (WNK is an acronym for "with no K", or lysine), whose variants lead to a reduced number of sensory neurons, although the exact mechanism for this is unknown. *KIF1A* encodes kinesin family member 1A, an axonal transporter of synaptic vescicles, and variants in this gene lead to impaired neuronal function. *RETREG1* encodes reticulophagy regulator 1, whose variants disrupt its physiological function in autophagy leading to neuronal toxicity and death. *WNK1*, *KIF1A*, and *RETREG1* variants lead to different subtypes of

HSAN, type II (57–62). Variants in *SCN9A* have also been implicated in HSAN, type II (63). *ELP1* encodes elongator complex protein 1, a scaffolding protein whose variants have been found in patients with HSAN, type III (64; 65). HSAN, type IV, as its alternative name – congenital insensitivity to pain with anhidrosis (CIPA) – suggests, is defined by insensitivity to pain as its primary symptom. Variants in *NTRK1*, which encodes neurotrophic receptor tyrosine kinase 1, disrupt its role in neuronal cell maintenance, thereby leading to CIPA (66–83). *NGF* encodes nerve growth factor beta, the binding partner of NTRK1. Its variants lead to HSAN, type V (84; 85). HSAN subtypes VII and VIII were characterised more recently, and their genetic causes have been determined to be variants in *SCN11A* (48; 49; 86) and *PRDM12* (87), respectively. A recent study by Chiabrando and colleagues showed variants in *FLVCR1*, a heme transporter, in a patient with an unclassified HSAN (88). All of these genes, in their pathological variant form, abolish pain sensitivity by depleting the number of viable sensory neurons.

B.3.2 Common disorders

Genetic studies of common neuropathic pain conditions suffer from a lack of clearly defined phenotyping (89). Despite the recently updated definition and grading system published by the neuropathic pain task force (1; 90; 91), this type of pain remains resistant to accurate diagnosis. While diabetic neuropathy, radicular pain, trigeminal neuralgia, and viral infection-related sensory neuropathies are among the more clearly defined neuropathic pain conditions, cancer pain and postoperative pain display mixed phenotypes of neuropathic and nociceptive pain. Many genetic reports do not adhere to standardised terminology and diagnostic procedures (92), and some studies report an association with chronic pain in cancer or post-surgery patients without characterising it. Nevertheless, given the substantial neuropathic component of these conditions we include these studies in our review.

Common neuropathic pain conditions, in which suspected genetic contribution derives from many different loci, benefit from studies in large cohorts with hypothesis-free scans of the entire genome. Thus, the publication of three genome-wide association studies (GWAS) in neuropathic pain during the past three years is an exciting recent development, even if the top findings in these studies are just below the threshold of genome-wide significance (93–95). Aside from these GWAS, studies done in patients genotyped or sequenced at targeted gene panels have been informative about loci that may modulate susceptibility to developing neuropathic pain following a traumatic event, physical injury, or the onset of another disease. Variants identified in this disease category are listed in Table B.1. The most fre-

quently investigated gene with variants reported to be associated with common neuropathic pain is *GCH1*.

Table B.1: Genetic variants reported in association studies of common neuropathic pain conditions. Abbreviations: DNP, diabetic neuropathic pain; PSP, postoperative pain; HIV-SN, HIV-related sensory neuropathy. Results from GWAS are in **boldface**.

Gene	SNP	Function/Pathway	Condition(s)	Citation
ABCB1	rs1045642	Pharmacokinetics	Cancer pain	(96)
CACNG2	rs4820242	Neurotransmission	PSP	(97)
	rs2284015	Neurotransmission	PSP	(97)
	rs2284017	Neurotransmission	PSP	(97)
CASP9	rs4645978	Apoptosis	Radicular pain	(98)
COL9A3	rs61734651	Structural	Radicular pain	(99)
COMT	rs4680	Neurotransmission	Cancer pain	(96)
			Herniated disc pain	(100)
DRD2	rs6277	Neurotransmission	Neuropathic pain	(101)
GCH1	rs8007267	Metabolism/Neurotransmission	Cancer pain	(102)
			HIV-SNP	(103; 104)
			PSP	(105; 106)
GCH1	rs8007201	Metabolism/Neurotransmission	PSP	(106)
GCH1	rs4411417	Metabolism/Neurotransmission	PSP	(106)
GCH1	rs752688	Metabolism/Neurotransmission	PSP	(106)
GCH1	rs10483639	Metabolism/Neurotransmission	Cancer pain	(102)
			HIV-SNP	(103; 104)
GCH1	rs3783641	Metabolism/Neurotransmission	Cancer pain	(102)
			HIV-SNP	(103; 104)
			PSP	(105; 106)
GFRA2	rs17428041	Immune response/Development	DNP	(93)
HMGB1P46	rs6986153	Unknown	DNP	(94)
IL10	rs3024505	Immune response	Postoperative pain	(107)
IL10	rs3024498	Immune response	Postoperative pain	(107)
IL10	rs3024496	Immune response	Postoperative pain	(107)
IL10	rs1878672	Immune response	Postoperative pain	(107)
IL10	rs1518111	Immune response	Postoperative pain	(107)
IL10	rs1518110	Immune response	Postoperative pain	(107)
IL10	rs3024491	Immune response	Postoperative pain	(107)
IL10RB	rs2834167	Immune response	Cancer pain	(108)
IL1A	rs1800587	Immune response	Radicular pain	(109; 110)
IL1B	rs1143627	Immune response	Cancer pain	(108)
IL1B	rs1143634	Immune response	Cancer pain	(111)
IL1R2	rs11674595	Immune response	Postoperative pain	(107)
IL1RN	rs2234677	Immune response	Radicular pain	(110)
IL6	rs1800797	Immune response	Radicular pain	(112; 113)
IL6	rs1800796	Immune response	Radicular pain	(112; 113)
IL6	rs1800795	Immune response	Radicular pain	(112; 113)
IL6	rs13306435	Immune response	Radicular pain	(112; 113)
KCNJ3	rs7574878	Neurotransmission	Cancer pain	(114)
KCNJ3	rs2591168	Neurotransmission	Cancer pain	(114)
KCNJ3	rs2591172	Neurotransmission	Cancer pain	(114)
KCNJ6	rs2835914	Neurotransmission	Cancer pain	(114)
KCNJ6	rs8129919	Neurotransmission	Cancer pain	(114)
KCNJ6	rs2836050	Neurotransmission	Cancer pain	(114)
KCNJ9	rs3780039	Neurotransmission	Cancer pain	(114)
KCNJ9	rs11166921	Neurotransmission	Cancer pain	(114)
KCNJ9	rs2014612	Neurotransmission	Cancer pain	(114)
KCNS1	rs734784	Neurotransmission	PSP	(115)
KCNS1	rs13043825	Neurotransmission	PSP	(115)
KCNS1	rs6017486	Neurotransmission	HIV-SN	(104)
KCNS1	rs6073643	Neurotransmission	HIV-SN	(104)
KCNS1	rs4499491	Neurotransmission	HIV-SN	(104)
			HIV-SN	(104)
LTA	rs1799964	Immune response	Cancer pain	(116)
MAPK1	rs8136867		Cancer pain	(117)
MAT2B/TENM2	rs7734804	Metabolism/Unknown	PSP	(95)
MMP1	rs1799750	Tissue remodelling	Radicular pain	(118)
NFKBIA	rs8904	Immune response	Cancer pain	(119)
NOS3	rs1800783	Neurotransmission	Cancer pain	(108)

OPRM1	rs1799971	Neurotransmission	DNP	(120)
			Postoperative pain	(121)
			Neuropathic pain	(122)
P2RX7	rs1718119	Immune system	DNP	(123)
P2RX7	rs208294	Immune system	DNP	(123)
	rs208294	Immune system	PSP	(124)
P2RX7	rs7958311	Immune system	PSP	(124)
PRKCA	rs887797	Cell signalling	PSP	(95)
PTGS2	rs5275	Immune response/Metabolism	Cancer pain	(108; 119)
PTGS2	rs5277	Immune response/Metabolism	Cancer pain	(116)
SCN9A	rs6746030	Neurotransmission	Peripheral neuropathy	(125)
	rs6746030	Neurotransmission	Radicular pain	(126)
SCN9A	rs3750904	Neurotransmission	DNP	(127)
SCN9A	rs4369876	Neurotransmission	DNP	(127)
SCN9A	rs200139913	Neurotransmission	DNP	(127)
SCN9A	rs74449889	Neurotransmission	DNP	(127)
TNF	rs28445017	Immune system	HIV-SN	(128)
TNF	rs1800629	Immune system	Cancer pain	(119)
TNFRSF1B	rs1061622	Immune response	Cancer pain	(108)
ZSCAN20	rs35260355	Unknown	DNP	(94)
ZSCAN20	rs71647933	Unknown	DNP	(94)

Diabetic neuropathy

Diabetic neuropathy is a condition of polar extremes, characterised by pain at one extreme and insensitivity at the other, and its prevalence is up to 50% in diabetes patients (129). Prolonged glycemic mismanagement and disruption of nerve microvasculature are the putative disease-associated risk factors (129; 130). However, given the incomplete penetrance of neuropathic pain in diabetic patients, the search for genetic contributors is ongoing. The first GWAS in the domain of painful neuropathies to be published was on diabetic neuropathic pain (93). Closely following came a second report from the same group (94). Both studies were conducted with the same cohort of almost 7000 genotyped diabetic patients. In the first study, cases of neuropathic pain were defined as individuals with a history of at least one specified diabetic peripheral neuropathy drug prescription and a positive monofilament test indicative of sensory neuropathy. In the second study, the monofilament test requirement was dropped, but cases had to have at least two prescriptions. The results in the first study showed a nearly genome-wide significant association for glial cell-derived neurotrophic factor, GDNF, family receptor alpha 2, encoded by *GFRA2*. In the second study the same level of significance was reported for a wide region in females on chr1p35.1, gated by zinc-finger and a conserved N-terminal motif, SCAN domain-encoding *ZSCAN20* on one end and toll-like receptor 12 pseudogene *TLR12P* on the other, and in males a high-mobility group box 1 pseudogene 46, *HMGB1P46*, on chr8p23.1.

Other groups have conducted association studies to examine the effects of *a priori* determined genetic variants, based on their roles in other related diseases. Among them, one has reported an association for the well-known A188G hypofunctional variant in μ-opioid receptor, *OPRM1*, with foot ulcer pain in diabetic patients (120). Another reported two hyperfunctional variants in purinergic receptor 7, *P2RX7*,

to be associated with higher pain in women diagnosed with diabetic neuropathy (123). Interleukin-4 receptor, encoded by *IL4R* has been reported to have its variable number of tandem repeats associated with diabetic neuropathy (131). Lastly, several hyperfunctional variants in sodium channel NaV1.7, *SCN9A*, were found in a cohort of nearly 1000 individuals with diabetes to be associated with neuropathic pain (127).

Radicular pain

Spinal disc herniation or prolapse leads to neuropathic pain through a combination of inflammation and nerve compression (132). Accompanying pain intensity and duration vary, and several studies have reported genetic variants as risk modifiers. Inflammatory mediators have shown association with herniated disc-related pain intensity, namely *IL1A* (109; 110); *IL1RN* (110); *IL6* (112; 113). Additionally, associations have been published for variants in *OPRM1* (122), *COMT* (100), *COL9A3* (encoding a chain of type IX collagen) (99), *MMP1* (encoding matrix metalloproteinase 1) (118), and *CASP9* (encoding caspase-9) (118).

Trigeminal neuralgia

Trigeminal neuralgia manifests as paroxysmal bursts of pain along the innervation pathway of the trigeminal nerve (133). According to recently proposed diagnostic criteria, its onset may be: 1) idiopathic, 2) due to an underlying condition, or 3) accompanying pressure exerted on the trigeminal nerve root by surrounding blood vessels (134). Genetic studies of trigeminal neuralgia have been scarce, with one report suggesting a variant in serotonin transporter, *SLC6A4*, (135) and a recent study suggesting sodium channel NaV1.6, encoded by *SCN8A*, (136). Both proteins are involved in neurotransmission and are suggestive of the nociceptive pathway. The finding of NaV1.6 is unique, because this is the first report of this channel's involvement in pain. Given its distribution in high-frequency firing neurons, it had previously been studied for its role in epilepsy (136).

Viral infection-related sensory neuropathies

Painful neuropathy as a sequela of the HIV infection is common and has been investigated by several groups in the recent years. An excellent review of the genetics of HIV-associated painful neuropathy on the African continent has just been published (137). Two genes harbouring variants associated with pain intensity in HIV-infected Southern Africans are *KCNS1* (104) and *TNF* (128; 138). Two groups have also investigated the involvement of *GCH1* in HIV-associated neuropathic pain in Africans but found no association (103; 104).

Postherpetic neuralgia is a condition characterised by persistent spontaneous or

innocuous-stimulus evoked pain. Several studies in Japanese patients have examined the role of genetic variants in the HLA region (139–142). Associations have been found for both class I molecules: *HLA-A*, *HLA-B*, and *HLA-C* (139–142); and class II *HLA-DRB1* (140; 141). The proposed mechanism whereby HLA complex variants contribute to postherpetic neuropathic pain is nerve damage permitted by inadequate immune system response to the initial viral infection (141).

Cancer pain

Up to 40% of all cancer pain has a neuropathic component (92). Aside from cancer-related surgery and chemotherapy, the cancer itself leads to neuropathic pain either by tumour invasion of nociceptors or by inflammatory cytokine leakage from cancerous cells (143). Given protracted inflammation, a sustained level of nociceptor activation could lead to persistent changes in neuronal connectivity, changing the response thresholds and intensities and transmitting innocuous stimuli as painful (119).

Variants in prostaglandin-endoperoxide synthase 2, *PTGS2*, tumour necrosis factor, *TNF*, and NFκB inhibitor-α *NFKBIA*, genotyped in (119), and tumour necrosis factor-β, *LTA*, genotyped in (116), have been reported to be associated with severe cancer pain. In another study, an aggregate of phenotypes that includes high pain intensity has been reported associated with the cumulative effect of variants in nitric oxide synthase-3, *NOS3*; interleukin-1β, *IL1B*; TNF receptor super-family member 1B, *TNFRSF1B*; *PTGS2*; and interleukin-10 receptor-β, *IL10RB* (108). Additionally, pain-protective variants in *GCH1*-encoded GTP cyclohydrolase, also involved in nitric oxide production, have been reported in patients with advanced cancer (102). These studies converge on a suggestive role for the immune system in modulating the extent of neuropathic pain accompanying cancer.

On the other hand, several studies have shown association with mediators of neurotransmission and even members of the pain-inhibition pathway. Variants in voltage-gated potassium ion channel-encoding *KCNS1*, *KCNJ3*, *KCNJ6*, and *KCNK9* have been reported in women with breast cancer pain prior to surgery (114), and variants in catechol-*O*-methyltransferase (*COMT*) and membrane-bounded P-glycoprotein (*ABCB1*) in charge of clearing exogenous opioids, have also been reported to be associated with pain in cancer patients (96).

Lastly, mitogen-activated protein kinase 1 (*MAPK1*) – a broad-spectrum regulator – has also been implicated in cancer pain (117).

Postoperative pain

Persistent postoperative pain is generally defined by the lower duration boundary of 2 to 6 months (144). Among putative causal mechanisms are nerve damage, which recruits immune cells, and a prolonged state of inflammation during the acute period (144; 145). In either case, inflammatory cytokine barrage leads to sustained nociceptor activity, which may result in a rewired pain transmission system (146–149).

Two studies of post-mastectomy patients, in whom neuropathic pain prevalence has been estimated to be up to 68% (150), reported associations between persistent breast pain and variants in interleukin-1 receptor, *IL1R2*, interleukin-10, *IL10* (107), and purinergic receptor 7, *P2RX7* (124).

Several variants in genes directly involved in neurotransmission have also been reported as risk modifiers in persistent postoperative pain, specifically μ-opioid receptor (*OPRM1*) in a post-abdominal surgery cohort (121), voltage-gated potassium channel subunit (*KCNS1*) in two limb amputation cohorts and one post-mastectomy cohort (115), and stargazin (*CACNG2*) – involved in the trafficking of AMPA receptors – in a post-mastectomy cohort (97). Variants in GTP hydrolase, encoded by *GCH1*, have been reported to modulate postoperative pain in two studies (105; 106).

The first genome-wide scan in a post-operative pain cohort (individuals with knee and hip replacement surgery) was published this year (95). The strongest associated variant, just shy of genome-wide significance (rs887797, P value = 1.29x10e-7), lies in *PRKCA*, which encodes protein kinase C alpha, and the next best association is for a variant in *MAT2B* (rs7734804, P value = 5.25x10e-6). Both of these associations were confirmed in one of their two replication joint-related neuropathic pain cohorts.

Other conditions

In several studies, neuropathic pain conditions were grouped into 1 phenotype, such that an association would indicate a link to condition-agnostic neuropathic pain. In one such study, a variant in dopamine receptor *DRD2* has been shown to be associated with susceptibility to pain given one of the following primary conditions: nerve injury, atypical facial pain burning mouth syndrome, and trigeminal neuropathy (101). Additionally, transient receptor potential channels, *TRPA1* and *TRPV1*, have been reported to affect somatosensory sensitivity in neuropathic pain patients who suffered from a variety of neuropathic conditions (151). A variant in *SCN9A* has also been reported to have association with pain by a group that examined five different cohorts with neuropathic pain (126).

Table B.2: Association with neuropathic pain in the UKBB cohort

SNP ID	Gene	Minor Allele	Effect (OR)	*P* value	FDR
rs1518110	IL10	C	1.1023	0.0013	0.0615
rs1518111	IL10	C	1.1005	0.0017	0.0615

FDR, false discovery rate; OR, odds ratio; SNP, single nucleotide polymorphism.

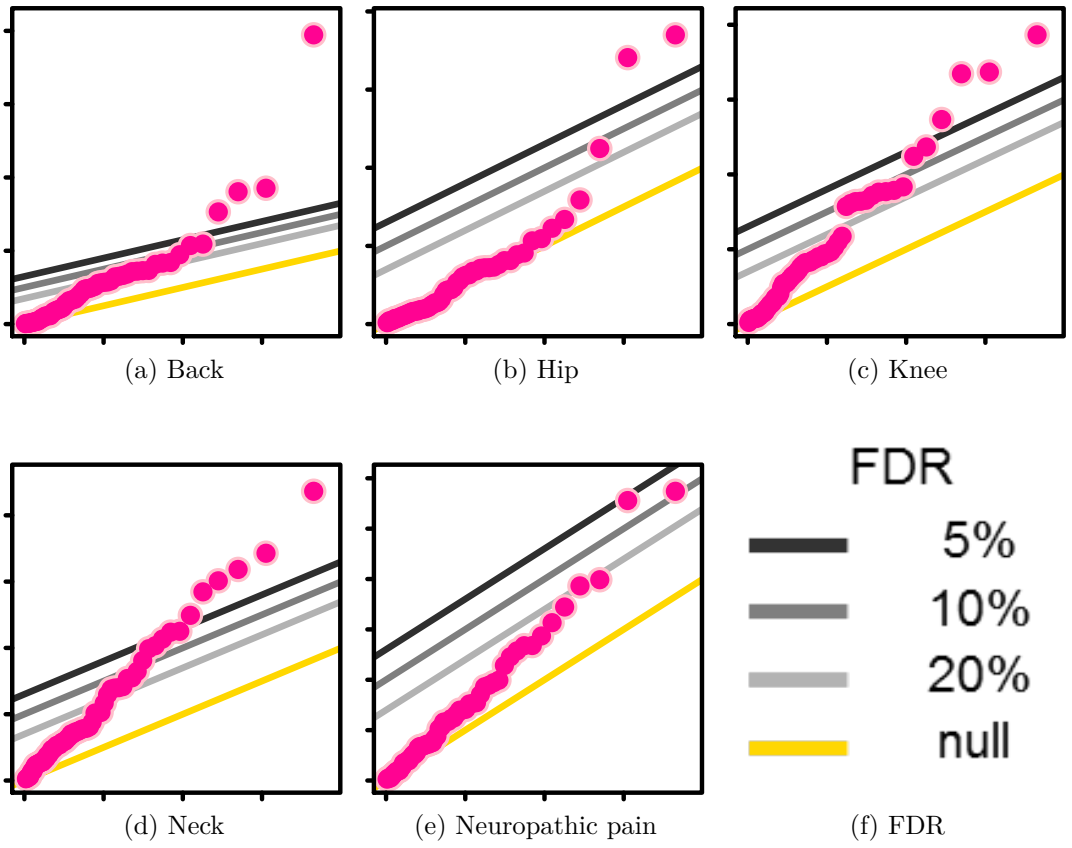
B.3.3 Replication in UKBB

We analysed the list of variants reported in association studies of common neuropathic pain conditions (Table B.1) for replication with neuropathic pain in UKBB. Associations for two single nucleotide polymorphisms (SNPs) on one haploblock of *IL10* pass correction for multiple testing at false discovery rate, FDR 20% (Table B.2). Previously reported in a post-operative pain cohort (107), these replicated associations, albeit nominal, give us increased confidence in the contribution of inflammatory mediators to neuropathic pain in a condition-agnostic manner, underscoring the importance of neuroimmune interactions already suspected to contribute to neuropathic pain (152; 153).

In addition, we tested the list of variants in Table B.1 for association with pain in four body sites – back, hip, knee, and neck – in all of which chronic pain may indicate neuropathy (154–158). Results of these association analyses are shown in quantile-quantile (QQ) plots (Figure B.3.3), which are a statistical tool to visualise the deviation of the observed distribution of association *P* values (log-transformed) from the one expected by chance, given uniform sampling in the *P* valuespace. Notably, while in all four sites several SNPs are associated with *P* values passing the FDR 5% threshold, SNP rs7734804, whose minor allele was originally reported as risk-conferring in a post-operative pain GWAS (95), is associated with the same direction of effect in all four sites and with back pain with genome-wide significance (*P* value = 1.3e-8 , OR=1.2), Table B.3.

B.4 Conclusion

This survey of literature provides an overview of genetic variants implicated in a variety of neuropathic pain conditions. Rare monogenic painful conditions are firmly rooted in ion channel – specifically sodium channel – mutations, underscoring the



(a) Back (b) Hip (c) Knee

(d) Neck (e) Neuropathic pain (f) FDR

Figure B.1: Results of association analysis for genetic variants reported in common neuropathic pain conditions (Table 1) with pain in different body sites (a-d) and neuropathic pain (e) in the UKBB cohort. In each QQ plot, the P values smaller than expected by chance surpass at least of the three fixed thresholds of statistical significance (false discovery rate, FDR = 5%, 10%, and 20%).

Table B.3: Association with pain in different body sites in the UKBB cohort

Type of pain	SNP ID	Gene	Minor Allele	Effect (OR)	*P* value	FDR
Back	rs7734804	MAT2B	T	1.20	1.3e-8	9.5e-7
	rs1800796	IL6	C	1.09	2.0e-4	6.0e-3
	rs6277	DRD2	A	0.96	2.4e-4	6.0e-3
	rs2591168	KCNJ3	A	0.96	8.7e-4	1.6e-2
Hip	rs7734804	MAT2B	T	1.16	2.0e-4	1.4e-2
	rs6277	DRD2	A	0.96	3.9e-4	1.4e-2
Knee	rs2591168	KCNJ3	A	0.96	1.4e-4	8.4e-3
	rs6277	DRD2	A	0.97	4.3e-4	8.4e-3
	rs7734804	MAT2B	T	1.11	4.5e-4	8.4e-3
	rs1295686	IL13	C	0.96	1.8e-3	2.0e-2
	rs2836050	KCNJ6	T	1.04	1.8e-3	2.0e-2
Neck	rs2591168	KCNJ3	A	0.95	4.3e-5	3.2e-3
	rs2836050	KCNJ6	T	1.05	3.7e-4	1.4e-2
	rs7734804	MAT2B	T	1.12	6.6e-4	1.6e-2
	rs1800796	IL6	C	1.08	9.7e-4	1.8e-2
	rs4411417	GCH1	C	0.97	1.4e-3	2.1e-2
	rs752688	GCH1	T	0.97	3.2e-3	4.0e-2

Associations with rs7734804 (upstream of MAT2B), highlighted in boldface, are in the same direction as in the original study. FDR, false discovery rate; OR, odds ratio; SNP, single nucleotide polymorphism.

critical role of these channels in pain processing. Among painless monogenic conditions, mutations disrupting nociceptive neuron maintenance are overrepresented. In common non-familial neuropathic pain conditions, the landscape of implicated molecules is more varied; the effect of genetic variants is considerably smaller and often harder to demonstrate. Nevertheless, neuroimmune interactions have emerged with a central role in neuropathic pain pathophysiology, supported by additional evidence from the UKBB study. While further studies are needed, this evidence supports the hypothesis that timely treatment targeting the immune system could be helpful in mitigating neuropathic pain. In addition, the involvement of neuropathic pain genetic variants in other pain conditions with a neuropathic pain component – in particular, a variant upstream of *MAT2B* whose association is prominent in back, hip, knee, and neck pain – provides preliminary evidence of shared contributing mechanisms at the genetic molecular level.

Diagnosing pain and confirming it as neuropathic in origin remains a challenge. The difficulty of identifying a nerve lesion or disease is exacerbated by other pain comorbidities and by the fact that diagnosis relies heavily on verbal interpretation of pain, far removed from the pathophysiological mechanisms that engender it. Thus, it is our hope that genetic studies will enable a more comprehensive assessment of

patients presenting with painful conditions and become a powerful tool in diagnosing and treating these conditions with requisite specificity.

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C

Appendix C: Common nonsynonymous variants in transient receptor potential, vanilloid 1 (*TRPV1*)

C.1 Introduction

Transient receptor potential vanilloid 1 cation channel, TRPV1, is a multimodal transducer of diverse noxious stimuli. It is expressed in the peripheral and central nervous systems, and it is activated by capsaicin, noxious heat ($> 45\,^{\circ}\mathrm{C}$), low extracellular pH, and a wide range of endogenous molecules. The signalling cascade initiated by TRPV1 activation is highly varied and context-dependent. TRPV1's tissue of expression, inflammation or disease state, receptor phoshorylation state, number of subunits with a bound ligand, duration and frequency of activation, as well as availability or intensity of the activating stimulus all contribute to the nature of the ensuing response and are able to direct whether TRPV1 activation will be perceived as painful or analgesic.

TRPV1 was originally identified as a heat-sensitive, vanilloid receptor (VR1), expressed in primary afferent C and Aδ fibres. Its activation results in a depolarising inward calcium current (1). It is best known for the painful burning sensation it elicits upon binding vanilloid moiety-carrying capsaicin, found in chili peppers. Repeated or excessive capsaicin administration leads to an initial period of hypersensitivity, followed by a prolonged period of reduced or abolished sensitivity and inflammation in response to chemical irritants (2). This phenomenon, discovered in animal models of inflammation in the mid 1900s, has been termed "capsaicin desensitisation" (3). The specific mechanism whereby this desensitisation is achieved has been under debate. Upon binding its receptor and activating it, the TRPV1 agonist lays out one of three possible courses of action: receptor desensitisation (4),

whole-neuron desensitisation (5), or neuron apoptosis (6; 7).

Perhaps more physiologically relevant than its response to a dietary irritant is TRPV1's role as mediator of injury-induced peripheral sensitisation (8). While its degree of activity under normal physiological conditions is undetectable, under inflammatory or neuropathic conditions, its membrane expression is increased and its activation threshold reduced, presumably to afford the injured or inflamed site time to heal unperturbed. This is supported by studies showing that *TRPV1*-knockout mice exhibit an unremarkable baseline phenotype but an absence of thermal hyperalgesia in post-inflammatory conditions (9). TRPV1 is furthermore responsive to a number of pro-inflammatory molecules: such as bradykinin, nerve-growth factor, arachidonic acid metabolites, and ATP (8). Given that TRPV1-positive peripheral nociceptors are bidirectional, they are both excited by these inflammatory mediators and release others, such as substance P and calcitonin gene-related peptide (CGRP), placing TRPV1 at the origin of neurogenic inflammation (8).

In addition to peripheral nociceptors, TRPV1 has been found in other pain-relevant neuronal populations (10; 11). One study reported this receptor's presence in the periaqueductal grey, where it initiates a signal cascade for the activation of descending inhibition cells in the rostral ventromedial medulla (10). Similarly, Kim and colleagues showed evidence of TRPV1 expression in spinal cord neurons, where they observed a pain transmission mechanism unrelated to peripheral nociceptor TRPV1 (11).

Given its role in inflammatory and thermal pain signalling, its responsiveness to a variety of stimulants, and its capsaicin-induced desensitisation, TRPV1 has been an attractive target for chronic pain treatment (3). However, its highly promiscuous and malleable agonist-binding profile has made it a challenge to establish whether pain inhibition is best accomplished by inhibiting it or activating it. Thus, both TRPV1 agonists and antagonists have been shown to be effective for chronic pain treatment (3; 9).

The encoding gene *TRPV1* was first cloned in 1997 by Caterina and colleagues (1). Since then, genetic techniques have been used to identify key amino acid residues in the TRPV1 protein for ligand binding and phosphorylation (12), and several genetic association studies have been published reporting statistical correlation between common missense genetic variants and chronic pain diseases. Specifically, the minor allele of rs222747 has been reported in (13) as protective in neuropathic pain patients. The major allele of rs8065080 has been reported to be associated with a lower risk of symptomatic knee osteoarthritis (14).

Less is known about the direct effects on acute pain sensitivity exercised by

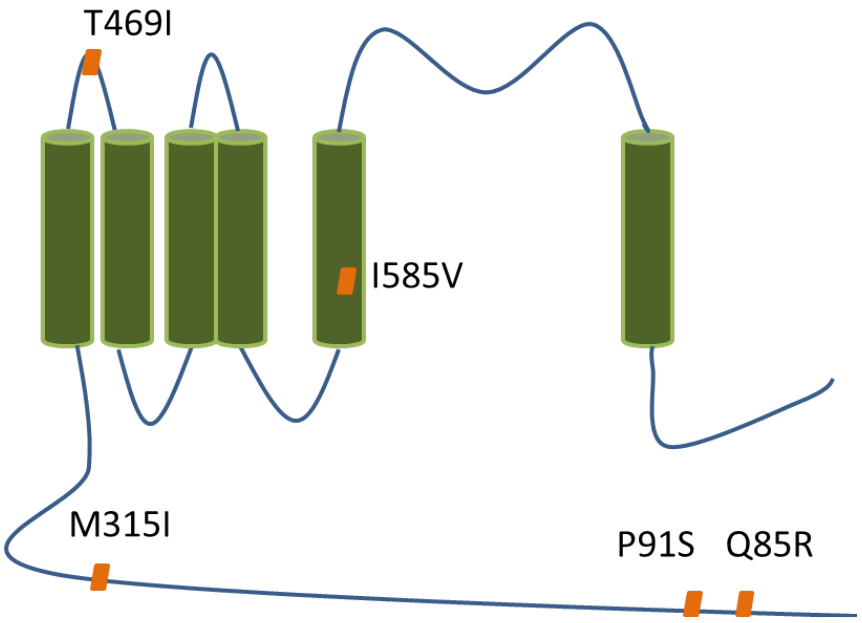


Figure C.1: Common missense variants in *TRPV1*.

common missense variants in the *TRPV1* gene that are at least 1 % prevalent in the population. There are 5 such variants: rs8065080 (I585V), rs224534 (T469I), rs222747 (M315I), rs222749 (P91S), and rs55916885 (Q85R), Figure C.1. Two studies done on experimental temperature-sensitive pain by the same group reported no associations for rs8065080 and rs55916885 with heat sensitivity (15; 16). Interestingly, rs8065080 was associated with reduced sensitivity to cold pain in female participants homozygous for the minor allele (15) and in another study with neuropathic pain (13).

A recent study undertook to characterise the *in vitro* cellular phenotype of these variants and determined 3 of them (rs224534, rs222749, and rs55916885) to confer gain of function, 1 loss (rs8065080), and 1 gain or loss (rs222747) depending on the specific function. By contrast, Xu and colleagues have shown that rs8065080 does not differ from wildtype in capsaicin response (17).

Here, we sought to interrogate the possible effect of all common missense *TRPV1* variants on the receptor's protective function, focusing on one of its main modes of activation – noxious heat. To characterise these variants as possible modulators of heat sensitivity, we aimed to query both individual variant association and their combined contribution by summing the count of variant alleles with the same direction of effect.

C.2 Methods

C.2.1 OPPERA cohort

The discovery cohort for this study was drawn from the Orofacial Pain Prospective Evaluation and Risk Assessment (OPPERA) project, designed and implemented according to the methods layed out in (18). Participants were recruited from the general population by advertisement at 4 U.S. study sites: the University of Maryland at Baltimore, the University of Buffalo (New York), the University of North Carolina at Chapel Hill, and the University of Florida at Gainesville. Of 4,353 participants, 1090 were diagnosed cases of temporomandibular disorder (TMD) upon recruitment. Genotyping was done at 2.5 million exonic single nucleotide polymorphism (SNP) positions on the Illumina Infinium Omni2.5Exome-8 panel, which comprehensively covers the protein content of the genome.

To test for association between *TRPV1* variants and pain sensitivity, we used quantitative sensory testing (QST). QST measures were collected as described in (19). Given TRPV1's role in thermal sensitivity, we tested its genetic variants for association with a panel of acute heat sensitivity measures. Briefly, a thermal stimulator was applied to each participant's forearm, and temperature was increased from a starting 32 °C at a rate of 0.5 °C/s until the participant reported it as painful (threshold) and subsequently intolerable (tolerance). The recorded temperature value for threshold and tolerance was an average of 4 trials, each done on a different part of the forearm. Thereafter, participants received 30 sequential suprathreshold thermal probe stimuli, divided into 3 sequences of 10 stimuli each, the first with a peak temperature of 46 °C, the second 48 °C, and the last at 50 °C. Recorded measures were individual stimulus ratings on a scale of 0-100, and 15-second and 30-second aftersensations following the 10th probe application in each series. From these ratings, derived measures used for analysis were: for each series of 10 the slope of the first 3 applications' ratings, the difference between the highest and the first stimulus rating, and the area under the curve of plotted ratings as a function of stimuli count.

C.2.2 Case-control TMD cohort

The case-control TMD cohort used for validation consisted of Caucasian females recruited at the University of North Carolina at Chapel Hill. 198 healthy controls and 200 TMD cases, diagnosed using the Research Diagnostic Criteria for TMD (20).

QST measures were collected as described in (21), following a similar protocol to the one used for the OPPERA cohort, except that instead of 3 series of summation stimuli 2 were applied, with cutoff temperatures 47C and 50C.

C.2.3 Statistical analysis

For initial determination of significant associations, we performed linear regression using a genetic association study web portal with a relational database structure (22), which incorporates PLINK software (23). Phenotype variable reduction was based on the results from principal component analysis of OPPERA heat measures, as described in (19) and for the case-control TMD cohort (21). Multiple testing correction was done according to the Holm-Bonferroni method (24; 25).

C.3 Results

First, we tested all 5 common missense variants for association with QST measures, designed to assess several different modes of sensitivity to heat. The results are reported in Table C.1. Given the multiple testing threshold of 0.0033, 3 variants – rs224534, rs55916885, and rs8065080 – had associations of statistical significance. Out of 7 significant associations, variants rs224534 and rs8065080 had 6 associations with aftersensation measures – pain ratings at 15 and 30-second intervals after the last of a series of 10 thermode applications. The direction of association is protective for both variants' minor alleles, although rs224534 confers gain of function and rs8065080 confers loss of function on TRPV1's cellular function (26). Additionally, the minor allele of rs55916885 is associated with the slope of the first 3 thermode application ratings in the risk direction.

Next we attempted to improve statistical power by combinining variants rs8065080 and rs222747, based on their shared effect in cellular response to heat as determined in (26). Collapsing their minor allele count into one variable, we analysed for its association with the top associated phenotype for rs8065080 in single-variant analysis – 50 °C 15 s after sensation. However, this association was not significant (P value = 0.1287).

The variants with significant associations were tested for validation in the TMD case-control cohort, with results reported in Table C.2. While rs224534 did not have any significant associations in this cohort, the associations for the other two variants were successfully replicated, with P values passing the multiple testing threshold of

Table C.1: Association results in OPPERA

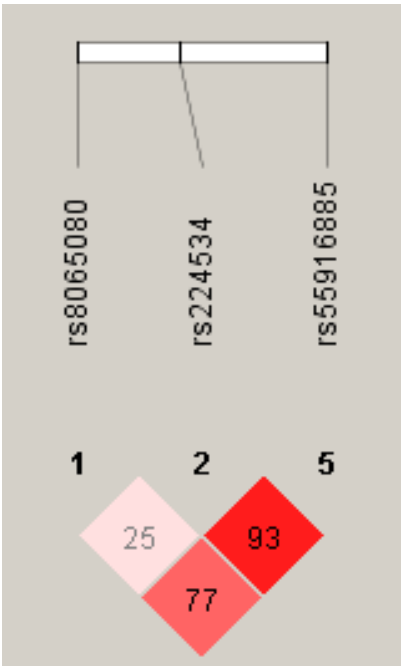
Variant	Minor Allele	Phenotype	Effect (β)	P value
rs224534	A	50 °C 15 s after sensation	-2.456	7.35e-5
rs224534	A	50 °C 30 s after sensation	-1.702	3.88e-4
rs224534	A	48 °C 30 s after sensation	-1.470	1.11e-3
rs224534	A	46 °C 30 s after sensation	-1.085	1.31e-3
rs224534	A	48 °C 15 s after sensation	-1.750	2.00e-3
rs55916885	G	50 °C slope	3.432	2.4e-3
rs8065080	G	50 °C 15 s after sensation	1.899	2.74e-3
rs222747	C	50 °C 15 s after sensation	-2.024	3.93e-3
rs55916885	G	50 °C delta	6.149	3.64e-3
rs8065080	G	50 °C 30sec after sensation	-1.288	8.47e-3
rs55916885	G	50 °C 15 s after sensation	-4.58	1.27e-2
rs222747	C	50 °C 30 s after sensation	-1.389	1.063e-2
rs8065080	G	48 °C 15 s after sensation	-1.451	1.258e-2

Table C.2: Association results in the replication cohort, COMT

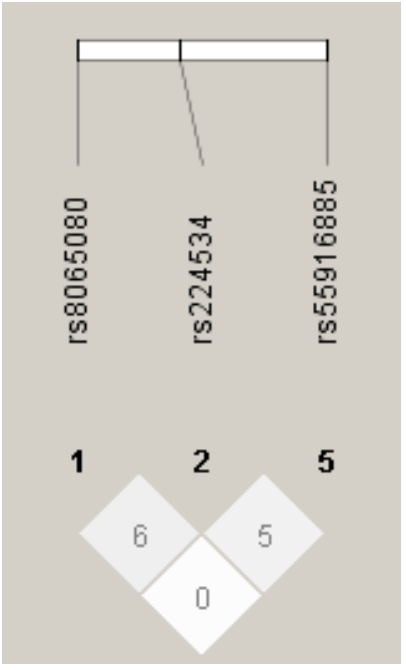
Variant	Minor Allele	Phenotype	Effect (β)	P value
rs8065080	G	47 °C trial 10	-6.531	2.953e-3
rs8065080	G	47 °C trial 9	-6.496	3.172e-3
rs8065080	G	47 °C trial 8	-5.899	2.953e-3
rs55916885	G	Tolerance	-1.064	6.471e-3
rs55916885	G	47 °C trial 8	15.75	7.696e-3
rs55916885	G	47 °C trial 7	15.66	8.249e-3

0.00833. The phenotypes in these associations are primarily single trial ratings, with 1 association for heat tolerance. The direction of effect is the same as in the OPPERA cohort: protective for rs8065080 and risk for rs55916885 (the negative β for tolerance also denotes risk, given that a lower tolerance implies more pain sensitivity.

Given that our discovery cohort associations were replicated for 2 of 3 variants and that these 2 variants had opposite functional cellular effects, we visualised the haplotype structure of these 3 variants in order to determine whether they worked cooperatively or in a compensatory manner. The linkage disequilibrium plot (LD), Figure C.2a shows that these 2 variants are indeed in high LD when measured using the allele-frequency-sensitive D' correlation metric. Given a wide discrepancy in the minor allele frequencies between the 2 variants, the value of the r-squared correlation metric is, of course, negligible, Figure C.2b.



(a)



(b)

Figure C.2: Linkage disequilibrium (LD) plot for 3 *TRPV1* variants. Correlation metric (number in the diamond): (a) D', (b) r-squared.

Haplotype	rs8065080	rs224534	rs55916885	Frequency
1	A	G	A	0.52
2	G	G	A	0.18
3	G	A	A	0.15
4	A	A	A	0.13
5	A	A	G	0.02

Table C.3: Haplotypes

In each haplotype the minor allele is highlighted in **boldface**.

C.4 Conclusion

Given *TRPV1*'s multimodal activation profile and its important role in pain signalling, understanding of its many nuanced modi operandi is critical for targetted pain treatment.

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