Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

An investigation of genetic polymorphisms in Heparan Sulfate Proteoglycan core proteins and key modification enzymes in an Australian Caucasian multiple sclerosis population

Rachel K Okolicsanyi
Queensland University of Technology
Julia Bluhm
Queensland University of Technology
Cassandra Miller
Queensland University of Technology
Lyn R Griffiths
Queensland University of Technology
Larisa M Haupt (larisa.haupt@qut.edu.au)
Queensland University of Technology https://orcid.org/0000-0002-7735-8110

Primary research

Keywords: Syndecans, glypicans, HSPG, Multiple Sclerosis, SNP, EXT1, SULF1

Posted Date: April 8th, 2020

DOI: https://doi.org/10.21203/rs.2.23090/v2

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License

Version of Record: A version of this preprint was published at Human Genomics on May 12th, 2020. See the published version at https://doi.org/10.1186/s40246-020-00264-6.

Abstract

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease affecting the central nervous system in young adults. Heparan sulfate proteoglycans (HSPGs) are ubiquitous to the cell surface and the extracellular matrix. HSPG biosynthesis is a complex process involving enzymatic attachment of heparan sulfate (HS) chains to a core protein. HS side chains mediate specific ligand and growth factor interactions directing cellular processes including cell adhesion, migration and differentiation. Two main families of HSPGs exist, the syndecans (SDC1-4) and glypicans (GPC1-6). The SDCs are transmembrane proteins, while the GPC family are GPI-linked to the cell surface. SDC1 has well-documented interactions with numerous signalling pathways. Genome wide association studies (GWAS) have identified regions of the genome associated with MS including a region on chromosome 13 containing GPC5 and GPC6. International studies have revealed significant associations between this region and disease development. The exostosin-1 (EXT1) and sulfatase-1 (SULF1) are key enzymes contributing to the generation of HS chains. EXT1, with documented tumour suppressor properties, is involved in initiation and polymerisation of the growing HS chain. SULF1 removes 6- O -sulfate groups from HS chains, affecting protein-ligand interactions and subsequent downstream signalling with HS modification potentially having significant effects on MS progression. In this study we identified significant associations between single nucleotide polymorphisms in SDC1, GPC5 and GPC6 and MS in an Australian Caucasian case control population. Further significant associations in these genes were identified when the population was stratified by sex and disease subtype. No association was found for EXT1 or SULF1.

Introduction

Multiple Sclerosis (MS) is one of the most common neurological diseases affecting young adults in Western society (Kutzelnigg, Lucchinetti et al. 2005). It is an inflammatory, demyelinating, neurodegenerative disease affecting the central nervous system (CNS) destroying myelin, oligodendrocytes, axons, and neurons (Noseworthy *et al*., 2000; Liguori *et al*., 2000). This disease is predominantly found in Caucasians with an onset between 18 and 40 years (Tajouri, Fernandez et al. 2007). Onset of MS varies from early childhood to adult life, affecting more than 25 000 people in Australia in 2017 (Ahmad, Palmer et al. 2018) and 2.3 million people worldwide in 2013 (Browne, Chandraratna et al. 2014). Symptoms include weakness in one or more limbs, visual disturbances and balance problems. As MS progresses, fatigue, bladder and bowel dysfunction, numbness, tremor, spasticity and/or depression may occur (Australia 2017). Individuals of Northern European ancestry, including those from Scandinavia, Iceland, the British Isles and North America, exhibit increased risk compared to African Americans (0.005%) (Kurtzke, Beebe et al. 1979, Zuvich, McCauley et al. 2009). There is a female predominance of about 3:1 (Gooch, Pracht et al. 2017).

MS is a complex genetic disease characterised by polygenic heritable components and multifaceted gene-environment interactions and factors (Oksenberg, Baranzini et al. 2008). Several studies have investigated the interaction between genes and environmental factors (Willer, Dyment et al. 2005) with underlying genetic factors implicated in determining familial clustering and individual susceptibility (Compston and Coles 2008). MS can be categorised into four subtypes: relapsing remitting MS (55%; RRMS); secondary progressive MS (30%; SPMS); primary progressive MS (10%; PPMS) and progressive relapsing MS (5%; PRMS) (Trapp and Nave 2008, Zuvich, McCauley et al. 2009). The severity and frequency of attacks as well as the reversibility of damage suffered during these attacks varies with disease state (Trapp and Nave 2008, Zuvich, McCauley et al. 2009). Females are twice as likely to develop RRMS as males (Rosati 2001, Trapp and Nave 2008) and due to the progressive axonal loss, 50% of RRMS cases convert to the late phase SPMS within 8 to 20 years (Zuvich, McCauley et al. 2009). The conversion to more severe forms of disease results in progressive disability and increasing irreversible damage with fewer remitting stages and no symptomatic relief (Huijbregts, Kalkers et al. 2004, Hauser and Oksenberg 2006, Huijbregts, Kalkers et al. 2006, Compston and Coles 2008, Zuvich, McCauley et al. 2009, Australia 2017).

Neurodegeneration is the major cause of permanent neurological disability in MS patients (Compston and Coles 2008). In the CNS neurons are composed of an oligodendrocyte axon surrounded by myelin, a lipid-rich insulating sheath (Brady, Witt et al. 1999) that allows rapid conduction of nerve impulses in the form of an action potential (Tajouri, Fernandez et al. 2007). Degradation of the myelin sheath, and hence axonal damage, resulting from deregulation of the immune system results in partial or complete blockage of CNS signal pathways (Compston and Coles 2008, Zuvich, McCauley et al. 2009). The formation of scar tissue (“sclerosis” or demyelinated plaques) in multiple locations within the CNS (Compston and Coles 2008, Zuvich, McCauley et al. 2009) produces symptoms common to MS including sensory disturbances (Hauser and Oksenberg 2006, Zuvich, McCauley et al. 2009). The mechanisms by which damage occurs in different subtypes is thought to differ, however, the outcome remains the same – primary demyelination and secondary axonal degeneration (Trapp and Nave 2008).

Early genetic studies associated the major histone compatibility (MHC) complex with MS and identified alleles of the human leukocyte antigen (HLA) region on chromosome 6q21 (6q21-23) common to MS (Olerup and Hillert 1991). Between 15-60% of the genetic aetiology of MS has been attributed to this region (Haines, Terwedow et al. 1998). A more recent strategy employed to identify genetic markers of MS is genome wide association studies (GWAS). These studies examine single nucleotide polymorphisms (SNPs) across the genome and provide additional genetic information about common complex disorders including identification of polymorphisms associated with diseases such as cancer and MS (Oksenberg and Baranzini 2010, 2011). In 2008 a GWAS identified non-HLA genes involved in MS (Oksenberg, Baranzini et al. 2008). Most of the genes identified were in immunological pathways (eg. Interleukin-7 receptor; (Xue, Kovanen et al. 2002, Gregory, Schmidt et al. 2007, International Multiple Sclerosis Genetics, Hafler et al. 2007, Lundmark, Duvefelt et al. 2007, Oksenberg, Baranzini et al. 2008, Baranzini, Wang et al. 2009) with confirmation of their impact gained through gene expression studies.

To date, the complex processes and factors that lead to demyelination, axonal damage, progressive neurological symptoms and the loss of immune homeostasis, remain poorly understood. However, during nervous system development the growing axons are thought to be guided towards their target by a complex that may include heparan sulfate proteoglycans (HSPGs) (Lee and Chien 2004).

HSPGs are found in both vertebrates and invertebrates (Cassaro and Dietrich 1977, Nader, Chavante et al. 1999) and are ubiquitous to the cell surface and the extracellular matrix (ECM). HSPGs belong to a large family of proteoglycans (PG) that are composed of one or more variably sulfated glycosaminoglycan chains (GAGs) attached to a core protein. Heparan sulfate (HS) chains undergo a complex, highly coordinated, sequential biosynthesis process resulting in a disaccharide chain consisting of *N*- and *O*-sulfated residues (Habuchi, Habuchi et al. 2004). This process involves a number of enzymes responsible for the initiation and subsequent modification of the growing GAG chain, resulting in a chain of variable length and sulfation pattern (Habuchi, Habuchi et al. 2004). Two of these enzymes are Exostosin-1 (EXT1) and Sulfatase-1 (SULF1). EXT1 encodes a glycotransferase protein that catalyses polymerisation of the GAG chain (Inatani and Yamaguchi 2003, Jennes, Zuntini et al. 2012). Complete abrogation of EXT1 results in embryonic lethality during gastrulation due to the absence of heparan sulfate (Lin, Wei et al. 2000, Holst, Bou-Reslan et al. 2007)

HSPGs interact and bind a variety of growth factors (eg. FGF-2, TGF-b), chemokines and morphogenes (Dreyfuss, Regatieri et al. 2009) to mediate downstream signalling and subsequent cellular processes. The ligand-binding properties of HS are associated with chain length (initiated by EXT1 and others), but more particularly, specific sulfation patterns conferred through the action of enzymes such as SULF1. SULF1 removes 6-*O*-sulfate groups, altering binding sites for signalling molecules (eg. FGF2) (Han, Huang et al. 2011) resulting in altered cell function. Mutations of SULF1 can result in a loss of function, potentially leading to lethality (Sahota and Dhoot 2009) while a double knockout of SULF1 results in neonatal lethality, similar to that seen in EXT1 knockouts (Nagamine, Tamba et al. 2012) although probably through a different mechanism.

When bound to the basal membrane, HSPGs mediate cell-cell and cell-ECM interactions (Lindahl, Kusche-Gullberg et al. 1998, Tumova, Hatch et al. 1999, Haupt and Griffiths 2009, Haupt, Murali et al. 2009) with the HS chains acting as receptors or co- receptors triggering cell responses such as proliferation, adhesion, migration and signalling (Filmus, Shi et al. 1995, Malavaki, Theocharis et al. 2011). Two major membrane bound families of HSPG core proteins are the four transmembrane syndecans (SDC; SDC1-4; (Tkachenko, Rhodes et al. 2005)) and the six glycosyl-phosphatidylinositol (GPI) anchored glypicans (GPC; GPC1-6; (David 1993, Filmus, Shi et al. 1995)). While SDC1 can carry other GAG chains, it predominantly carries HS chains (Bernfield, Gotte et al. 1999) and is a known binding partner of FGF-2 (Ling, Murali et al. 2006). GPCs carry only HS chains with attachment points close to the cell surface (Filmus, Capurro et al. 2008). They are known to have a role in cell proliferation and differentiation and interact with the Wnt signalling pathway. In addition, GPCs have also been suggested to have a role in the inflammatory response (Lorentzen, Melum et al. 2010).

One promising non-immune region, not previously identified in MS genome linkage screens, was 13q31 (Comabella, Craig et al. 2008). One GWAS analysed over 500 000 SNPs in 978 MS cases and 883 control samples and identified the HSPG GPC5 as a novel candidate gene for increased risk in MS (Baranzini, Wang et al. 2009). A follow-up study genotyped an additional 974 MS affected individuals with similar results obtained for the GPC5 region (Baranzini, Wang et al. 2009). A subsequent study investigating the GPC5 and GPC6 genes in susceptibility to MS determined their strongest association to be a specific GPC5 SNP (rs9523762) (Lorentzen, Melum et al. 2010).

With the genetics of MS not completely understood, genes found to have a modest effect may provide potential new markers of therapy. Previous studies have identified GPCs in active MS plaques (Cavanillas, Fernandez et al. 2011) and have associated these genes with disease. In contrast, little research has been conducted to elucidate the genetic involvement of SDCs, or HS chain modification enzymes, in MS development and progression. However, there is sufficient evidence to suggest involvement of these genes through their participation in cellular signalling. Indeed, FGF-2, a known signalling partner of the SDCs, has a key role in the regulation of differentiation and proliferation and therefore may have a role in remyelination (Ling, Murali et al. 2006).

To further investigate the role of HSPGs in MS susceptibility, this study set out to replicate previously identified GWAS SNP associations (GPC5, GPC6, 13q31-32 (Comabella, Craig et al. 2008)), and to extend our knowledge of the potential involvement of a number of related genes in an Australian Caucasian case-control population. We investigated polymorphisms within HS chain modification enzymes, EXT1 and SULF1, as well as those within HSPG core proteins SDC1, GPC5 and GPC6. Disruption to the critical enzymes responsible for the diverse functions of the HS side chains, or the HSPG core proteins themselves, could have an adverse effect on the progression of MS.

Methods

Population DNA

This study utilised a Caucasian (Northern European descent) case-control population. The population consisted of 205 multiple sclerosis (MS) patient samples and 194 age (+/- 5 years)and sex matched control samples. The case samples were then further divided into three disease states: relapsing-remitting (RR), secondary progressive (SP) and primary progressive (PP) MS. The case group consisted of 160 females and 45 males. A summary of the numbers of males and females in each group can be found in Table 1. Genomic DNA was extracted from peripheral blood using a standard salting-out procedure described previously (Tajouri, Mellick et al. 2005; Griffith Ethics Approval: 1300000488; QUT Ethics Approval: 1400000502).

DNA was analysed for quantity and quality using the Nanodrop (Thermo Scientific, Australia). Where necessary, samples were cleaned using standard ethanol precipitation protocols and reanalysed for quantity and quality. Samples were diluted to a working concentration of 20ng /µL. Aliquots were stored at -20°C.

Primers

Primers for EXT1, SULF1 and SDC1 were designed using NCBI primer blast (NCBI). Primers for GPC5 and GPC6 were designed using PyroMark Assay Design Software v2.0 (Qiagen, Australia) for use in pyrosequencing and subsequent analysis. For GPC6, rs9524260 a sequencing primer was included in the design process. Primer sequences can be found in Table 2. Primers were synthesised by Integrated DNA Technologies (IDT), USA.

Polymerase Chain Reaction (PCR)

For EXT1 and SULF1 40ng of DNA was amplified with 1X PCR buffer, 100nM each forward and reverse primers (IDT, USA), 200 µM dNTPs (NEB, Australia), 1.75mM $MgCl_2$, 0.5U GoTaq Flexi DNA Polymerase (Promega, Australia) in a 15µL reaction. Optimal reaction conditions for SDC1 amplified 40ng of DNA with 1X PCR buffer, 200nM each forward and reverse primers, 200 µM dNTPs, 1.75mM $MgCl_2$, 0.5U GoTaq Flexi DNA Polymerase in a 15µL reaction.

Annealing temperatures (T_a) for individual assays can be found in Table 2. Cycling conditions for these three SNPs were as follows: an initial denaturation step at 95^{o}C for 10 minutes was followed by 35 cycles of 95^{o}C for 30 seconds, annealing at T_a for 30 seconds and extension at 72^{o}C for 30 seconds. This was followed by a final extension step at 72^{o}C for 5 minutes.

PCR products for all SNPs were analysed on a 2% agarose gel in 1X TAE with ethidium bromide at 90V for at least 30min to confirm amplification of single PCR products of the correct size. A 100bp ladder was used alongside the samples for sizing purposes. Fragments were visualised using UV light.

Restriction Fragment Length Polymorphism (RFLP)

Genotypes for EXT1, SULF1 and SDC1 were determined by RFLP. EXT1 PCR product (7µL, approximately 1µg DNA) was digested with 0.5U Cac8I enzyme, 1X NEB reaction buffer 4 in a total volume of 15uL. Digestion occurred overnight at 37^{o}C followed by an inactivation step at 65^{o}C for 20min. Digest products were then analysed on a 3% agarose gel in 1X TAE with ethidium bromide at 90V for 45min. DNA fragments were visualised under UV light. A 100bp ladder was used alongside digest products for sizing purposes. The enzyme Cac8I recognises the sequence GCNNGC. The presence of the wild-type G allele allows for digestion producing fragments of 281bp and 115bp. The homozygous variant (AA) remained uncut with a single band at 397bp.

SULF1 and SDC1 PCR products (7µL, approximately 1µg DNA) were digested with 1U PspGI enzyme, 1X NEB reaction buffer 4 in a 15µL reaction. Samples were digested at 75^{o}C for 16hrs. No inactivation step was required. SULF1 digest products were analysed on a 3% agarose gel as for EXT1. SDC1 digest products were assessed on a 4% agarose gel in 1X TAE with ethidium bromide at 75V for 1hr along with a 100bp ladder for sizing purposes. Fragments were visualised under UV light. The enzyme PspGI recognises the sequence CCWGG. The digestion of SULF1 PCR products produces bands at 212bp and 78bp in the presence of the wild-type C allele while the homozygous variant remained uncut with a single band visible at 291bp. When PCR product from SDC1 was digested, bands were produced at 188bp, 37bp and 17bp regardless of the allele present. In the presence of the wild-type G allele bands are also produced at 53bp and 43bp, while the homozygous variant (CC) contains a single extra band at 96bp.

High Resolution Melt

High resolution melt (HRM) analyses were performed on the Rotor Gene™ 6000 (Corbett, Australia), Rotor-Gene® Q (Qiagen, Australia) or with the 7900 HT Fast Real-Time PCR System (Applied Biosystems, Australia). All DNA case-control samples were assayed in duplicate. For each SNP, positive control samples were identified from samples not included in the analysis population. Where possible, positive controls representing each of the three genotypes were identified for each SNP.

HRM reaction conditions amplified 40ng of DNA with 1X reaction buffer, 1.75mM $MgCl_2$, 100nm each of forward and reverse primers, 100nM dNTPs, 50µM Syto®9 and 0.5U GoTaq Hot Start DNA Polymerase in a 15uL reaction. Cycling conditions on the ABI 7900 HT system included an initial 2 min hold at 50^{o}C followed by denaturation and HotStart polymerase activation step of 95^{o}C for 10 min. This was followed by 40 cycles of 95^{o}C for 15s and T_a for 1 min. Finally, products were melted between 60^{o}C-90^{o}C to produce the melt curves required to differentiate between genotypes. The Rotor Gene 6000 system utilised the following cycling conditions: denaturation and HotStart polymerase activation at 95^{o}C for 10 min followed by 40 cycles of 95^{o}C for 5s, T_a for 10s, and a final melt between 70^{o}C – 90^{o}C. The ABI 7900 HT system was used to genotype rs7333912 and rs9523787 (GPC5) while the Rotor Gene 6000 system was used for genotyping rs10492503 (GPC5) and rs17267815 (GPC6).

Pyrosequencing

Pyrosequencing was performed as per the manufacturer's instructions on the Pyromark Q24 (Qiagen, Australia). Briefly, 15µL of PCR product was combined with 2µL of Streptavidin Sepharose beads (GE Healthcare, Australia), 40µL of binding buffer (10mM Tris-HCl, 2M NaCl, 1mM EDTA, 0.1% Tween^{TM} 20, pH7.6) in a reaction volume of 80µL. Amplicons were denatured (denaturation buffer: 0.2M NaOH) and washed with washing buffer (10mM Tris-Acetate) and 70% ethanol. The clean biotin-labelled amplicons were transferred to a sequencing plate containing 0.3µM sequencing primer diluted in annealing buffer (20mM Tris-Acetate, 5mM $MgAc_2$) and denatured for 2min at 80^{o}C. Samples were loaded into the PyroMark Q24 chamber for analysis. The reagent cartridge was loaded with appropriate volumes of dNTPs and Enzyme and Substrate mix (Qiagen) as determined by proprietary Q24 equipment software.

Sequencing Validation

To verify the results obtained from the HRM genotyping represent the three different genotypes, positive controls and examples of each genotype from the population samples were sequenced. This was performed by Sanger sequencing using the BigDye® Terminator (BDT) v3.1 Cycle sequencing kit (Thermo Fisher Scientific, Australia). Briefly, PCR product was cleaned with ExoSAP-IT® (Affimetrix) following manufacturers protocols. PCR product concentration was estimated and adjusted for optimal sequencing conditions. Following the BDT reaction, the samples were then cleaned using a standard ethanol precipitation method, dried and resuspended in water. Forward and reverse reactions for each sample were prepared. Samples were then loaded into a 96 well plate followed by separation on a four capillary 3130 genetic analyser system (Thermo Fisher Scientific, Australia). The results were later analysed with AB Sequencing Analysis Software v5.3.

Statistical analysis

Genotype and allele frequencies were calculated using a standard counting method. Populations were analysed by Hardy-Weinberg equilibrium (HWE) and Chi-square tests. A significance level of $p<0.05$ was used. Where allele or genotype analysis was significant, the population was stratified by either disease type or sex and reanalysed. Global p-values were adjusted for multiple testing using the Benjimini-Hocherg and Bonferroni methods. Corrections were conducted in R v3.3.0 and Rstudio v0.99.896. Corrected p-values are presented in results tables.

For the GPC5 and GPC6 SNPs within the chromosome 13 risk region, linkage Disequilibrium (LD) analysis was preformed using Haploview v4.2.

Results

We examined eight single nucleotide polymorphisms (SNPs) within HSPG initiation and modification enzyme (*EXT1* and *SULF1)* and HSGP core protein (*SDC1*, *GPC5* and *GPC6)* genes in an Australian case-control population for their role in MS susceptibility. Genotype and allele frequencies were also compared to results from the HapMap CEU reference population. The chromosomal region of 13q31-32, where GPC5 and GPC6 are located, has been previously identified in a GWAS as showing a significant association with genetic susceptibility of multiple sclerosis (MS). We aimed to investigate the role of these genes as well as the potential role of enzymes responsible for the modification of the HS chains attached to these and other core proteins. This study investigated eight SNPs in five genes. Differences in final population numbers successfully genotyped for each SNP analysis are due to variation in assay efficiency as well as DNA availability.

Modification Enzymes

EXT1 and SULF1 enzymes initiate and modify HS side chains attached to core proteins. The action of these enzymes determines the final length and sulfation pattern of the side chain, and mediate signalling interactions (eg. growth factors). They are critical for HSPG synthesis and any mutation could result in an autosomal dominant disorder (Jennes, Zuntini et al. 2012).

EXT1, rs11546829

No significant association was found between the rs11546829 SNP in EXT1 and MS. Both case and control populations followed HWE and allele and genotype frequencies matched the HapMap CEU reference population. When the population was stratified by disease type and further analysed there was no association found with disease state. Results are summarised in Table 3.

SULF1, rs2623047

No significant association was found between rs2623047 in SULF1 and MS. HWE was observed in both case and control populations. Allele and genotype frequencies matched the HapMap CEU reference population. Further analysis of the stratified populations revealed no significant association with disease type. Results are summarised in Table 4.

Core Proteins

Syndecan-1

SDC1 is known to have a role in various cancers, including breast cancer (Okolicsanyi, 2013) through its role in cell adhesion, migration and proliferation. Through its interaction with FGF-2, SDC1 has also been proposed to have a role in remyelination (Ling, Murali et al. 2006). Evidence

suggests that SDC1 may be associated with MS through enhanced expression of TGF-β in MS lesions that may lead to increased expression of SDC1 (van Horssen, Bo et al. 2006). A link between SDCs and the innate immune response has also been postulated as these HSPGs have been observed in injured tissues, regulating the accompanying inflammatory response (Bernfield, Gotte et al. 1999), suggesting a link to the inflammatory response seen in MS patients.

SDC1, rs1131351

A positive association between the rs1131351 SNP in SDC1 and MS was identified. Both case and control populations followed HWE. When compared to the HapMap CEU reference population allele and genotype frequencies were similar. Allele and genotype frequencies showed significant differences between the case and control populations. When the population was examined by disease state, significant association for the SDC1 SNP was observed at the allelic level for RRMS and PPMS for the whole population. There was no significant association with SPMS. Results are summarised in Table 5.

The population was further analysed by sex, where SDC1 showed a further significant association in the female MS population (Table 5). The female population was further stratified by disease state. A significant association was observed in females with PPMS at both the genotype and allelic level and female RRMS cases at the allelic level only with no observed significance with genotypes. The female SPMS group demonstrated no significant association at either allele or genotype level and MS. Results are summarised in Table 6.

Glypican-5

The three GPC5 SNPs (rs7333912, rs10492503 and rs9523787) investigated in this study have previously been significantly associated with MS in Caucasian European populations (Cenit, Blanco-Kelly et al. 2009, Lorentzen, Melum et al. 2010). Another GPC5 SNP (rs9523762) not reported here, was found to be positive in one study (Baranzini, Wang et al. 2009) while another study identified moderate LD between it and rs9523787 but did not find it to be individually significant (Lorentzen, Melum et al. 2010). Indeed, in this current study we also investigated this SNP but have not presented the results as analysis revealed significant deviation from HWE. As this Australian population consists of Caucasian ancestors, we performed an associative study with these SNPs in an Australian Caucasian population to see if the association could be replicated in this cohort. Differences between our results and previous studies may be due to the more mixed heritage of the Australian Caucasian population compared with the purer northern European Caucasian populations previously examined.

GPC5, rs7333912

The GPC5 SNP, rs7333912, is an intergenic SNP, located at 13q31-32 approximately 150,000 bp upstream of the GPC5 gene on chromosome 13. This variation is a C/G polymorphism with no homozygous variants (GG) observed either in our population or in the HapMap CEU reference population. The case and control populations were found to be in HWE with no significant association identified between this SNP and MS. When the population was stratified and analysed by disease state, no significant association was found with disease type. Results are summarised in Table 7.

GPC5, rs10492503

GPC5-rs10492503 is located in the same intron as rs9523787 (intron7-8) within the 13q31-32 MS susceptibility locus that has previously been associated with MS (Comabella, Craig et al. 2008). In our study, both case and control populations observed HWE. A significant association was identified between this SNP and disease at both the genotype and allele level. When further analysed by disease state, further significant associations were determined in the SPMS group at the allelic level and RRMS case group at the both the genotypic and allelic level. Stratification by sex determined a significant association between this SNP and the female case group at both genotype and allele level. Results are summarised in Table 8. The female case group was further analysed by disease state. A significant association was found at both genotypic and allelic level between the SNP and female SPMS and RRMS disease subtypes. These results are summarised in Table 9.

GPC5, rs9523787

This variation is located toward the 3' end of the gene in intron 7-8, the same intron that contains rs10492503 (Comabella, Craig et al. 2008). GPC5-rs9523787 had previously been associated with MS and to be in modest LD with a variation located close by, rs9523762 (Lorentzen, Melum et al. 2010). We were unable to replicate either of these findings. Both case and control populations followed HWE and we found no significant association between this variation and the MS population. Populations stratified by disease type also showed no significant association. Results are summarised in Table 10.

Glypican-6

GPC6 has previously been implicated in MS (Lorentzen, Melum et al. 2010). In that study, 22 SNPs were analysed with only three showing a significant association with disease (GPC5-rs7333912, GPC6-rs17267815, GPC6-rs12876985). Only GPC5-rs7333912 and GPC6-rs17267815 were included in this study. The second GPC6 SNP investigated in this study was previously reported to be associated with Primary Sclerosing Cholanigitis (PSC) (Karlsen, Franke et al. 2010). GPC6 is located in the chromosome region of 13q31 neighbouring the risk region identified in genome wide screens (Comabella, Craig et al. 2008).

GPC6, rs17267815

Of 22 SNPs analysed by Lorentzen and colleagues, this SNP (GPC6-rs17267815) showed the greatest significance (Lorentzen, Melum et al. 2010) with MS. In our study no significant association was identified in the total case group versus controls. However, when the cases were analysed by disease state, we found a significant association between the RRMS case group and the SNP at both the genotype and allelic level. Results are summarised in table 11. There was no significance when stratified by sex. However, when the population was stratified by both disease and sex, an association was found at both the genotype and allelic level for the male RRMS subgroup. This is, however, only suggestive as the sample numbers for the homozygous variant genotype fell below the minimum required for reliable Chi-square testing (n<5). These results are summarised in Supplementary Table 1.

GPC6, rs9524260

Located within the 13q31 risk region, rs9524260 is an intronic SNP. Both case and control populations were found to be in HWE with no significant association found between this variation and MS. There was also no significant association when the population was stratified by disease type or sex. Results are summarised in Table 12.

LD Analysis of GPC5 and GPC6 SNPs

GPC5 and GPC6 markers were analysed for LD using Haploview v4.2. No LD was observed in this population. The highest D' value determined was between rs10492503 and rs9523787 in GPC5 with D'=0.15. The LD plot can be seen in Figure 1.

Discussion

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) with presumed autoimmune origin, triggered by genetic and environmental risk factors. The aetiology of MS is unknown, and the pathology is not well understood. In addition to those SNPs identified as significant in a 2009 GWAS (Baranzini, Wang et al. 2009) we investigated two SNPs in enzymes responsible for the initiation and modification of the side chains characteristic of HSPGs (EXT1, SULF1) and another HSPG core protein, SDC1. No significant difference was observed in our MS population in relation to the EXT1 and SULF1 SNPs examined. Further analysis revealed no association with disease subtype. However, in this study we did identify significant associations with SDC1, GPC5 and GPC6 polymorphisms. Overall, analysis by disease subtype maintained this significance, as did analysis by sex.

While this study may suggest that no association exists between EXT1 and MS, previous studies have indicated strong expression of EXT1 in the developing brain (Inatani and Yamaguchi 2003). Additionally, it has been suggested that EXT1 correlates with the sites of active neuron generation (Inatani and Yamaguchi 2003). Prenatally EXT1 has been localised in the neuroepithelial cells, which surround the lateral ventricles, cerebral cortex and hippocampus. However, in the postnatal stage EXT1 is expressed in the cerebellum, which may correlate with the symptoms seen in MS such as ataxia (Inatani and Yamaguchi 2003). In a murine model complete abrogation of EXT1 results in embryonic lethality during gastrulation due to the absence of HS (Holst, Bou-Reslan et al. 2007). EXT1 alone is able to polymerise GAG chains for attachment to PG core proteins (Busse and Kusche-Gullberg 2003, Kim, Kitagawa et al. 2003), however, both EXT1 and EXT2 are required for *in vivo* HS chain elongation (Busse-Wicher, Wicher et al. 2014).

SULF1 has been suggested to have a role in the brain, however deficiencies in SULF1 have been associated with developmental abnormalities such as decreased body mass and subtle kidney and bone defects (Holst, Bou-Reslan et al. 2007). SULF1 has also been linked to tumour suppressor functions as it has been reported to be downregulated in some cancers (Han, Huang et al. 2011).

The process of HS chain biosynthesis requires the action of enzymes such as SULF1 to generate complex sulfation patterns through addition and removal of sulfation sites. Successful binding of growth factors to GAG chains for signalling pathway activation requires specific sulfation patterns on these side chains. The interaction between HS and FGF-2 is a well-documented example where HS requires 2-*O* sulfation to be able to bind to FGF-2. Without this binding, cellular proliferation and differentiation are inhibited. Although, these two SNPs in EXT1 and SULF1 showed no significance in our population of moderate size, further investigation should be undertaken with a larger cohort before excluding the possibility of their involvement in MS susceptibility.

In the first of the HSPG core proteins examined (SDC1) we found a significant association between the SNP, rs1131351, and MS. This association revealed a stronger link between the SNP and females suffering from early onset forms of disease (PPMS, RRMS). SPMS occurs 8-20 years after RRMS onset (Trapp and Nave 2008) with the negative association seen here with this disease state suggestive of a role for SDC1 in the initiation of disease. Females with PPMS and the minor allele of SDC1 have more than double the risk (OR=2.24) of developing MS than controls. In patients suffering from RRMS this increase in risk is approximately 1.5 times (OR=1.57). This could be due to the fact that PPMS seems to be more aggressive during onset when compared with RRMS. Even though they are both classified as onset stages of the disease, RRMS can progress to SPMS, with reversible damage occurring in this stage, while PPMS damage is irreversible and the symptoms are generally more detrimental (reviewed in (Goldenberg 2012, Dutta and Trapp 2014).

Active MS lesions are characterised by an influx of inflammatory cells and a decrease of chondroitin sulfate proteoglycans (CSPGs) (van Horssen, Bo et al. 2006). Furthermore, white matter-associated PGs have been known to accumulate in macrophages, suggesting that CSPGs are phagocytosed with myelin or their breakdown products (van Horssen, Bo et al. 2006). SDC1 contains ser-gly sequences that may serve as an attachment site for chondroitin sulfate (CS) (Bernfield, Gotte et al. 1999) while also carrying HS chains. A mutation in SDC1 may contribute to activation of the macrophages causing phagocytosis, subsequently leading to a reduction in SDC1 in MS patients. In addition, TGF-β along with FGF-2, have been linked to enhanced expression of SDC1 (Bernfield, Gotte et al. 1999). Enhanced expression of TGF-β has been observed in MS lesions causing matrix deposition by the promotion of transcription genes and suppression of degrading enzymes (van Horssen, Bo et al. 2006). FGF-2 has been associated with the survival, proliferation and migration of oligodendrocyte precursors leading to the promotion of remyelination (van Horssen, Dijkstra et al. 2007). This contradicts the mechanism of neurodegeneration seen in MS patients however, FGF-2 could be a survival mechanism established to reverse the damage particularly in relapsing and remitting MS patients, through its binding partners other than SDC1.

In addition to the SDC1-FGF-2/TGF- β signalling mechanisms, TNF-α has been demonstrated to decrease SDC1 expression in cultured endothelial cells (Bernfield, Gotte et al. 1999). TNF-α has been shown to be involved in the inflammatory response (Titelbaum, Degenhardt et al. 2005) and could be involved in the process mimicking the early stages of MS where breakdown of the blood brain barrier allows inflammatory cells to cross into the brain and contribute to demyelination and axonal damage (van Horssen, Bo et al. 2006).

In this study we aimed to replicate and build on results from a number of previous GWAS and replication studies using an Australian case-control population. These earlier results implicated GPC5 and GPC6 SNPs in MS. Our analysis of three GPC5 and two GPC6 SNPs also identified significant associations between these genes and MS susceptibility. GPC5-rs10492503 showed a significant association in the total disease population. When analysed further we found significant associations with two disease states (SPMS and RRMS) and in the total female population and the female SPMS and RRMS subtypes. GPC6-rs17267815 showed a minor significant association within the RRMS subtype only. Further analysis suggested this association was due to the male RRMS subgroup, however due to low sample numbers once the population was stratified, significance values are suggestive only.

We identified no LD between the SNPs studied within the previously identified 13q31-32 risk region containing both these genes, nor could we replicate the moderate LD identified previously in GPC5 (Lorentzen, Melum et al. 2010). All five GPC5 and GPC6 SNPs investigated in this study had previously been identified as significant in large-scale case/control GWAS and replication studies in Norwegian and Spanish populations (Comabella, Craig et al. 2008, Baranzini, Wang et al. 2009, Cenit, Blanco-Kelly et al. 2009, Lorentzen, Melum et al. 2010) with varying and often contradictory levels of significance. SNPs reaching significance in one study were not found to be significant in another (Baranzini, Wang et al. 2009, Lorentzen, Melum et al. 2010, Cavanillas, Fernandez et al. 2011). Analysis by disease state of some of these populations determined significant associations with the RRMS subtype (Poliseno, Salmena et al. 2010). Indeed, in our population, when a significant association was observed in these genes, it was often significant in the RRMS sub-population. This may be due to the mixture of the populations as patients from pure Northern European ancestry have a higher risk of developing MS (Kurtzke, Beebe et al. 1979). While our Australian population is of Caucasian decent, it is not necessarily of purely Northern European origin, potentially explaining some differences between results and levels of significance identified in these studies. In addition, while our results are not strongly significant on their own, they replicate previous studies and support and strengthen the evidence for involvement of GPC5 and GPC6 in the development and progression of MS.

Many HSPGs and their associated enzymes have been associated with disease, with both SDC1 and SDC4 showing strong involvement with breast cancer (Tkachenko, Rhodes et al. 2005, Lendorf, Manon-Jensen et al. 2011, Okolicsanyi, van Wijnen et al. 2014). As yet, the physiological functions of the GPCs, in both normal and pathological conditions, remains poorly understood. However, data here and in other studies suggest an important function for these proteins in cell growth and regulation of division. Celie and colleagues suggest that HSPGs are involved in the inflammatory response and have a regulatory role in leukocyte extravasation (Celie, Beelen et al. 2009), a condition synonymous with MS. Other GPCs have been shown to play roles in diseases such as hepatocellular carcinoma (GPC3; (Capurro, Xiang et al. 2005)) and Simpson-Golabi-Behmel syndrome (GPC3/GPC4; (Veugelers, De Cat et al. 1999)). While the function of *GPC5* remains poorly understood, particularly in MS, different GPC polymorphisms have been reported to increase the risk of lung cancer in non-smokers (Li, Sheu et al. 2010) while decreasing the risk of cancer in MS patients, with this reduced cancer risk stemming from the specific GPC gene (Handel and Ramagopalan 2010). In addition, the gene region 13q31-32 containing both GPC5 and GPC6 has also previously been associated with increased risk of Primary Sclerosing Cholangitis (PSC), a chronic liver disease where a strong association has been identified between the SNP GPC6-rs9524260 and disease (Karlsen, Franke et al. 2010).

Due to the interaction of the GPCs with several growth factors, chemokines and ECM proteins, there may also be an effect on neural growth and repair (Byun, Caillier et al. 2008). The results of a study by Cenit and colleagues (2009) not only supported a significant association of GPC5-rs10492503 with MS, but also indicated approximately twice the risk of developing disease in an individual who has one or more copies of the variant allele (Cenit, Blanco-Kelly et al. 2009). GPC5 has been reported to play an important role during the process of cell division and growth regulation. It is predominantly expressed in foetal tissues, including brain, lung, liver and kidney. However, it has an exclusive expression in adult tissue in the CNS and in its neurons (Saunders, Paine-Saunders et al. 1997, Veugelers, De Cat et al. 1999). This suggests a possible and plausible role for this gene in controlling various neurotropic factors and maintenance of neural function. In our study we found a significant association of this GPC5 variation with the early onset from of disease (RRMS) and also the severe form (SPMS), a progression of disease characterised by irreversible damage suggesting a role for GPC5 in the progression of MS. GPC5 plays an important role in brain patterning, synapse formation, axon regeneration and

guidance. Its expression in the in the developing brain and the adult CNS (the origin of MS) also support a role for this gene in different disease states.

In PPMS most of the myelin degradation occurs in the cerebrum and cerebellar cortex of the CNS (Kutzelnigg, Lucchinetti et al. 2005). Dysfunction of GPC5 could affect cell proliferation and tissue growth. With the cells no longer able to interact with the various positively charged growth factors, this would affect brain patterning, synapse formation, and an interruption in axon regeneration. This suggests, that abnormal *GPC5* may play a role in triggering MS and the subsequent disability experienced by sufferers.

Further evidence supporting this hypothesis is data demonstrating that HSPGs have been identified in the active lesions of MS, where they are thought to be involved in the sequestering of pro-inflammatory chemokines (van Horssen, Bo et al. 2006). GPC5 expression and interaction with various growth factors and chemokines likely affects growth and repair of neurons, also influencing the guidance of axons and synapse formation (Lee and Chien 2004, van Horssen, Bo et al. 2006, Van Vactor, Wall et al. 2006). Indeed, another member of the GPC family - GPC1, has been shown to be required for Schwann cell myelination (Chernousov, Rothblum et al. 2006). With documented involvement of other GPCs, it is plausible allelic variants of GPC5 may affect neuronal repair, axon guidance and new synaptic formation.

The embryonic expression of GPC6 is detected in the ovary, liver and kidneys, while in the adult, it is detected only in the ovary and intestine (Fransson 2003). More recent emerging evidence for GPC6 may indicate a role for the gene in neural diseases with origins in the CNS, the location of the MS associated lesions. Overall, the functional role for GPC6 is poorly understood, but this study provides some evidence of a potential role for GPC6 in MS.

Conclusion

From this study we have determined a significant association with the rs1131351 SNP in SDC1, specifically in females suffering from either primary progressive or relapsing remitting forms of MS. Involvement of SDC1 in the initiation of MS has been suggested through its involvement in the inflammatory response and growth factor interactions. Levels of specific growth factors may vary during MS onset which could be due to dysfunction of HSPGs brought about by their inability to appropriately traffic / sequester growth factors. The specific mechanism of GPC5 and GPC6 involvement in MS has yet to be elucidated. However, a number of genetic studies, this one included, have provided evidence suggesting a role for these genes in the progression of disease. Evidence already exists for these genes in other diseases utilising similar mechanisms of action. Significant results obtained in this study have been summarised in Figure 2. This schematic highlights the involvement of specific SNPs in specific disease states as well as whether the SNP is associated with either the male or female subpopulation. Results from this study are by no means conclusive, but they add to the growing body of evidence indicating involvement of these PGs in the initiation and progression of neurodegenerative diseases. Specifically, this study supports and strengthens evidence suggesting a role for HSPG core proteins, both syndecans and glypicans, in the development and progression of MS.

Declarations

Ethics Approval:

Ethical approval for these studies is through Queensland University of Technology (QUT) Human Research Ethics Committee HREC (Approval Number 1400000502) and previously through Griffith University Human Research Ethics Committee (1300000488).

Consent for Publication:

Not applicable.

Availability of Data and Materials:

All data relevant for this study is included within this manuscript, any further information may be made available on request.

Competing Interests:

The authors declare that they have no competing interests regarding the information in this manuscript.

Funding:

This work was supported by infrastructure purchased with Australian Government EIF Super Science Funds as part of the Therapeutic Innovation Australia - Queensland Node project. RKO was supported by and Australian Postgraduate Award and the authors wish to acknowledge the support of the Estate of the late Clem Jones AO (LMH, LRG).

Author's Contributions:

Conception and design of the manuscript was completed by RKO and LMH. Design and completion of genotyping assays was conducted by RKO, JB and CM. Analysis of HRM melt curves, RFLP fragment separation and pyrosequencing traces was conducted by RKO, JB and CM. Writing and editing of the manuscript was completed by RKO with substantial editing contributed by LMH and LRG. All authors approved submission of the manuscript to *Human Genetics*.

Acknowledgements:

Not applicable

References

(2011). "National Human Genome Research Institute." Retrieved 18 January, 2012, from http://www.genome.gov/27544865.

Ahmad, H., A. Palmer, C. JA, I. van der Mei and B. Taylor (2018). Health Economic Impact of Multiple Sclerosis in Australia in 2017. Hobart, Tasmania, Menzies Institute for Medial Research.

Australia, M. S. S. o. (2017). "Symptoms." 2019, from https://www.msaustralia.org.au/about-ms/symptoms.

Baranzini, S. E., J. Wang, R. A. Gibson, N. Galwey, Y. Naegelin, F. Barkhof, E. W. Radue, R. L. Lindberg, B. M. Uitdehaag, M. R. Johnson, A. Angelakopoulou, L. Hall, J. C. Richardson, R. K. Prinjha, A. Gass, J. J. Geurts, J. Kragt, M. Sombekke, H. Vrenken, P. Qualley, R. R. Lincoln, R. Gomez, S. J. Caillier, M. F. George, H. Mousavi, R. Guerrero, D. T. Okuda, B. A. Cree, A. J. Green, E. Waubant, D. S. Goodin, D. Pelletier, P. M. Matthews, S. L. Hauser, L. Kappos, C. H. Polman and J. R. Oksenberg (2009). "Genome-wide association analysis of susceptibility and clinical phenotype in multiple sclerosis." Hum Mol Genet **18**(4): 767-778.

Bernfield, M., M. Gotte, P. W. Park, O. Reizes, M. L. Fitzgerald, J. Lincecum and M. Zako (1999). "Functions of cell surface heparan sulfate proteoglycans." Annu Rev Biochem **68**: 729-777.

Brady, S. T., A. S. Witt, L. L. Kirkpatrick, S. M. de Waegh, C. Readhead, P. H. Tu and V. M. Lee (1999). "Formation of compact myelin is required for maturation of the axonal cytoskeleton." J Neurosci **19**(17): 7278-7288.

Browne, P., D. Chandraratna, C. Angood, H. Tremlett, C. Baker, B. V. Taylor and A. J. Thompson (2014). "Atlas of Multiple Sclerosis 2013: A growing global problem with widespread inequity." Neurology **83**(11): 1022-1024.

Busse, M. and M. Kusche-Gullberg (2003). "In vitro polymerization of heparan sulfate backbone by the EXT proteins." J Biol Chem **278**(42): 41333-41337.

Busse-Wicher, M., K. B. Wicher and M. Kusche-Gullberg (2014). "The exostosin family: proteins with many functions." Matrix Biol **35**: 25-33.

Byun, E., S. J. Caillier, X. Montalban, P. Villoslada, O. Fernandez, D. Brassat, M. Comabella, J. Wang, L. F. Barcellos, S. E. Baranzini and J. R. Oksenberg (2008). "Genome-wide pharmacogenomic analysis of the response to interferon beta therapy in multiple sclerosis." Arch Neurol **65**(3): 337-344.

Calboli, F. C., F. Tozzi, N. W. Galwey, A. Antoniades, V. Mooser, M. Preisig, P. Vollenweider, D. Waterworth, G. Waeber, M. R. Johnson, P. Muglia and D. J. Balding (2010). "A genome-wide association study of neuroticism in a population-based sample." PLoS One **5**(7): e11504.

Capurro, M. I., Y. Y. Xiang, C. Lobe and J. Filmus (2005). "Glypican-3 promotes the growth of hepatocellular carcinoma by stimulating canonical Wnt signaling." Cancer Res **65**(14): 6245-6254.

Cassaro, C. M. and C. P. Dietrich (1977). "Distribution of sulfated mucopolysaccharides in invertebrates." J Biol Chem **252**(7): 2254-2261.

Cavanillas, M. L., O. Fernandez, M. Comabella, A. Alcina, M. Fedetz, G. Izquierdo, M. Lucas, M. C. Cenit, R. Arroyo, K. Vandenbroeck, I. Alloza, M. Garcia-Barcina, A. Antiguedad, L. Leyva, C. L. Gomez, J. Olascoaga, D. Otaegui, Y. Blanco, A. Saiz, X. Montalban, F. Matesanz and E. Urcelay (2011). "Replication of top markers of a genome-wide association study in multiple sclerosis in Spain." Genes Immun **12**(2): 110-115.

Celie, J. W., R. H. Beelen and J. van den Born (2009). "Heparan sulfate proteoglycans in extravasation: assisting leukocyte guidance." Front Biosci (Landmark Ed) **14**: 4932-4949.

Cenit, M. D., F. Blanco-Kelly, V. de las Heras, M. Bartolome, E. G. de la Concha, E. Urcelay, R. Arroyo and A. Martinez (2009). "Glypican 5 is an interferon-beta response gene: a replication study." Mult Scler **15**(8): 913-917.

Chernousov, M. A., K. Rothblum, R. C. Stahl, A. Evans, L. Prentiss and D. J. Carey (2006). "Glypican-1 and alpha4(V) collagen are required for Schwann cell myelination." J Neurosci **26**(2): 508-517.

Comabella, M., D. W. Craig, M. Camina-Tato, C. Morcillo, C. Lopez, A. Navarro, J. Rio, M. S. S. G. Biomarker, X. Montalban and R. Martin (2008). "Identification of a novel risk locus for multiple sclerosis at 13q31.3 by a pooled genome-wide scan of 500,000 single nucleotide polymorphisms." PLoS One **3**(10): e3490.

Compston, A. and A. Coles (2008). "Multiple sclerosis." Lancet **372**(9648): 1502-1517.

David, G. (1993). "Integral membrane heparan sulfate proteoglycans." FASEB J **7**(11): 1023-1030.

Dreyfuss, J. L., C. V. Regatieri, T. R. Jarrouge, R. P. Cavalheiro, L. O. Sampaio and H. B. Nader (2009). "Heparan sulfate proteoglycans: structure, protein interactions and cell signaling." An Acad Bras Cienc **81**(3): 409-429.

Dutta, R. and B. D. Trapp (2014). "Relapsing and progressive forms of multiple sclerosis: insights from pathology." Curr Opin Neurol **27**(3): 271-278.

Filmus, J., M. Capurro and J. Rast (2008). "Glypicans." Genome Biol **9**(5): 224.

Filmus, J., W. Shi, Z. M. Wong and M. J. Wong (1995). "Identification of a new membrane-bound heparan sulphate proteoglycan." Biochem J **311 (Pt 2)**: 561-565.

Fransson, L. A. (2003). "Glypicans." Int J Biochem Cell Biol **35**(2): 125-129.

Goldenberg, M. M. (2012). "Multiple sclerosis review." P T **37**(3): 175-184.

Gooch, C. L., E. Pracht and A. R. Borenstein (2017). "The burden of neurological disease in the United States: A summary report and call to action." Ann Neurol **81**(4): 479-484.

Gregory, S. G., S. Schmidt, P. Seth, J. R. Oksenberg, J. Hart, A. Prokop, S. J. Caillier, M. Ban, A. Goris, L. F. Barcellos, R. Lincoln, J. L. McCauley, S. J. Sawcer, D. A. Compston, B. Dubois, S. L. Hauser, M. A. Garcia-Blanco, M. A. Pericak-Vance, J. L. Haines and G. Multiple Sclerosis Genetics (2007). "Interleukin 7 receptor alpha chain (IL7R) shows allelic and functional association with multiple sclerosis." Nat Genet **39**(9): 1083-1091.

Habuchi, H., O. Habuchi and K. Kimata (2004). "Sulfation pattern in glycosaminoglycan: does it have a code?" Glycoconj J **21**(1-2): 47-52.

Haines, J. L., H. A. Terwedow, K. Burgess, M. A. Pericak-Vance, J. B. Rimmler, E. R. Martin, J. R. Oksenberg, R. Lincoln, D. Y. Zhang, D. R. Banatao, N. Gatto, D. E. Goodkin and S. L. Hauser (1998). "Linkage of the MHC to familial multiple sclerosis suggests genetic heterogeneity. The Multiple Sclerosis Genetics Group." Hum Mol Genet **7**(8): 1229-1234.

Han, C. H., Y. J. Huang, K. H. Lu, Z. Liu, G. B. Mills, Q. Wei and L. E. Wang (2011). "Polymorphisms in the SULF1 gene are associated with early age of onset and survival of ovarian cancer." J Exp Clin Cancer Res **30**: 5.

Handel, A. E. and S. V. Ramagopalan (2010). "GPC5 and lung cancer in multiple sclerosis." Lancet Oncol **11**(8): 714.

Haupt, L. M. and L. R. Griffiths (2009). "Heparan Sulfate Proteoglycans, Tumour Progression and the Cancer Stem Cell Niche." Current Cancer Therapy Reviews **5**(4): 256-260.

Haupt, L. M., S. Murali, F. K. Mun, N. Teplyuk, L. F. Mei, G. S. Stein, A. J. van Wijnen, V. Nurcombe and S. M. Cool (2009). "The heparan sulfate proteoglycan (HSPG) glypican-3 mediates commitment of MC3T3-E1 cells toward osteogenesis." J Cell Physiol **220**(3): 780-791.

Hauser, S. L. and J. R. Oksenberg (2006). "The neurobiology of multiple sclerosis: genes, inflammation, and neurodegeneration." Neuron **52**(1): 61-76.

Holst, C. R., H. Bou-Reslan, B. B. Gore, K. Wong, D. Grant, S. Chalasani, R. A. Carano, G. D. Frantz, M. Tessier-Lavigne, B. Bolon, D. M. French and A. Ashkenazi (2007). "Secreted sulfatases Sulf1 and Sulf2 have overlapping yet essential roles in mouse neonatal survival." PLoS One **2**(6): e575.

Huijbregts, S. C., N. F. Kalkers, L. M. de Sonneville, V. de Groot and C. H. Polman (2006). "Cognitive impairment and decline in different MS subtypes." J Neurol Sci **245**(1-2): 187-194.

Huijbregts, S. C., N. F. Kalkers, L. M. de Sonneville, V. de Groot, I. E. Reuling and C. H. Polman (2004). "Differences in cognitive impairment of relapsing remitting, secondary, and primary progressive MS." Neurology **63**(2): 335-339.

Inatani, M. and Y. Yamaguchi (2003). "Gene expression of EXT1 and EXT2 during mouse brain development." Brain Res Dev Brain Res **141**(1-2): 129-136.

International Multiple Sclerosis Genetics, C., D. A. Hafler, A. Compston, S. Sawcer, E. S. Lander, M. J. Daly, P. L. De Jager, P. I. de Bakker, S. B. Gabriel, D. B. Mirel, A. J. Ivinson, M. A. Pericak-Vance, S. G. Gregory, J. D. Rioux, J. L. McCauley, J. L. Haines, L. F. Barcellos, B. Cree, J. R. Oksenberg and S. L. Hauser (2007). "Risk alleles for multiple sclerosis identified by a genomewide study." N Engl J Med **357**(9): 851-862.

Jennes, I., M. Zuntini, K. Mees, A. Palagani, E. Pedrini, G. De Cock, E. Fransen, W. Vanden Berghe, L. Sangiorgi and W. Wuyts (2012). "Identification and functional characterization of the human EXT1 promoter region." Gene **492**(1): 148-159.

Karlsen, T. H., A. Franke, E. Melum, A. Kaser, J. R. Hov, T. Balschun, B. A. Lie, A. Bergquist, C. Schramm, T. J. Weismuller, D. Gotthardt, C. Rust, E. E. Philipp, T. Fritz, L. Henckaerts, R. K. Weersma, P. Stokkers, C. Y. Ponsioen, C. Wijmenga, M. Sterneck, M. Nothnagel, J. Hampe, A. Teufel, H. Runz, P. Rosenstiel, A. Stiehl, S. Vermeire, U. Beuers, M. P. Manns, E. Schrumpf, K. M. Boberg and S. Schreiber (2010). "Genome-wide association analysis in primary sclerosing cholangitis." Gastroenterology **138**(3): 1102-1111.

Kim, B. T., H. Kitagawa, J. Tanaka, J. Tamura and K. Sugahara (2003). "In vitro heparan sulfate polymerization: crucial roles of core protein moieties of primer substrates in addition to the EXT1-EXT2 interaction." J Biol Chem **278**(43): 41618-41623.

Kurtzke, J. F., G. W. Beebe and J. E. Norman, Jr. (1979). "Epidemiology of multiple sclerosis in U.S. veterans: 1. Race, sex, and geographic distribution." Neurology **29**(9 Pt 1): 1228-1235.

Kutzelnigg, A., C. F. Lucchinetti, C. Stadelmann, W. Bruck, H. Rauschka, M. Bergmann, M. Schmidbauer, J. E. Parisi and H. Lassmann (2005). "Cortical demyelination and diffuse white matter injury in multiple sclerosis." Brain **128**(Pt 11): 2705-2712.

Lee, J. S. and C. B. Chien (2004). "When sugars guide axons: insights from heparan sulphate proteoglycan mutants." Nat Rev Genet **5**(12): 923-935.

Lendorf, M. E., T. Manon-Jensen, P. Kronqvist, H. A. Multhaupt and J. R. Couchman (2011). "Syndecan-1 and syndecan-4 are independent indicators in breast carcinoma." J Histochem Cytochem **59**(6): 615-629.

Li, Y., C. C. Sheu, Y. Ye, M. de Andrade, L. Wang, S. C. Chang, M. C. Aubry, J. A. Aakre, M. S. Allen, F. Chen, J. M. Cunningham, C. Deschamps, R. Jiang, J. Lin, R. S. Marks, V. S. Pankratz, L. Su, Y. Li, Z. Sun, H. Tang, G. Vasmatzis, C. C. Harris, M. R. Spitz, J. Jen, R. Wang, Z. F. Zhang, D. C. Christiani, X. Wu and P. Yang (2010). "Genetic variants and risk of lung cancer in never smokers: a genome-wide association study." Lancet Oncol **11**(4): 321-330.

Lin, X., G. Wei, Z. Shi, L. Dryer, J. D. Esko, D. E. Wells and M. M. Matzuk (2000). "Disruption of gastrulation and heparan sulfate biosynthesis in EXT1-deficient mice." Dev Biol **224**(2): 299-311.

Lindahl, U., M. Kusche-Gullberg and L. Kjellen (1998). "Regulated diversity of heparan sulfate." J Biol Chem **273**(39): 24979-24982.

Ling, L., S. Murali, C. Dombrowski, L. M. Haupt, G. S. Stein, A. J. van Wijnen, V. Nurcombe and S. M. Cool (2006). "Sulfated glycosaminoglycans mediate the effects of FGF2 on the osteogenic potential of rat calvarial osteoprogenitor cells." J Cell Physiol **209**(3): 811-825.

Lorentzen, A. R., E. Melum, E. Ellinghaus, C. Smestad, I. L. Mero, J. H. Aarseth, K. M. Myhr, E. G. Celius, B. A. Lie, T. H. Karlsen, A. Franke and H. F. Harbo (2010). "Association to the Glypican-5 gene in multiple sclerosis." J Neuroimmunol **226**(1-2): 194-197.

Lundmark, F., K. Duvefelt, E. Iacobaeus, I. Kockum, E. Wallstrom, M. Khademi, A. Oturai, L. P. Ryder, J. Saarela, H. F. Harbo, E. G. Celius, H. Salter, T. Olsson and J. Hillert (2007). "Variation in interleukin 7 receptor alpha chain (IL7R) influences risk of multiple sclerosis." Nat Genet **39**(9): 1108-1113.

Malavaki, C. J., A. D. Theocharis, F. N. Lamari, I. Kanakis, T. Tsegenidis, G. N. Tzanakakis and N. K. Karamanos (2011). "Heparan sulfate: biological significance, tools for biochemical analysis and structural characterization." Biomed Chromatogr **25**(1-2): 11-20.

Nader, H. B., S. F. Chavante, E. A. dos-Santos, T. W. Oliveira, J. F. de-Paiva, S. M. Jeronimo, G. F. Medeiros, L. R. de-Abreu, E. L. Leite, J. F. de-Sousa-Filho, R. A. Castro, L. Toma, I. L. Tersariol, M. A. Porcionatto and C. P. Dietrich (1999). "Heparan sulfates and heparins: similar compounds performing the same functions in vertebrates and invertebrates?" Braz J Med Biol Res **32**(5): 529-538.

Nagamine, S., M. Tamba, H. Ishimine, K. Araki, K. Shiomi, T. Okada, T. Ohto, S. Kunita, S. Takahashi, R. G. Wismans, T. H. van Kuppevelt, M. Masu and K. Keino-Masu (2012). "Organ-specific sulfation patterns of heparan sulfate generated by extracellular sulfatases Sulf1 and Sulf2 in mice." J Biol Chem **287**(12): 9579-9590.

Okolicsanyi, R. K., A. J. van Wijnen, S. M. Cool, G. S. Stein, L. R. Griffiths and L. M. Haupt (2014). "Heparan sulfate proteoglycans and human breast cancer epithelial cell tumorigenicity." J Cell Biochem **115**(5): 967-976.

Oksenberg, J. R. and S. E. Baranzini (2010). "Multiple sclerosis genetics–is the glass half full, or half empty?" Nat Rev Neurol **6**(8): 429-437.

Oksenberg, J. R., S. E. Baranzini, S. Sawcer and S. L. Hauser (2008). "The genetics of multiple sclerosis: SNPs to pathways to pathogenesis." Nat Rev Genet **9**(7): 516-526.

Olerup, O. and J. Hillert (1991). "HLA class II-associated genetic susceptibility in multiple sclerosis: a critical evaluation." Tissue Antigens **38**(1): 1-15.

Poliseno, L., L. Salmena, J. Zhang, B. Carver, W. J. Haveman and P. P. Pandolfi (2010). "A coding-independent function of gene and pseudogene mRNAs regulates tumour biology." Nature **465**(7301): 1033-1038.

Rosati, G. (2001). "The prevalence of multiple sclerosis in the world: an update." Neurol Sci **22**(2): 117-139.

Sahota, A. P. and G. K. Dhoot (2009). "A novel SULF1 splice variant inhibits Wnt signalling but enhances angiogenesis by opposing SULF1 activity." Exp Cell Res **315**(16): 2752-2764.

Saunders, S., S. Paine-Saunders and A. D. Lander (1997). "Expression of the cell surface proteoglycan glypican-5 is developmentally regulated in kidney, limb, and brain." Dev Biol **190**(1): 78-93.

Tajouri, L., F. Fernandez, S. Tajouri, G. Detriche, A. Szvetko, N. Colson, P. Csurhes, M. P. Pender and L. R. Griffiths (2007). "Allelic variation investigation of the estrogen receptor within an Australian multiple sclerosis population." J Neurol Sci **252**(1): 9-12.

Tajouri, L., A. S. Mellick, A. Tourtellotte, R. M. Nagra and L. R. Griffiths (2005). "An examination of MS candidate genes identified as differentially regulated in multiple sclerosis plaque tissue, using absolute and comparative real-time Q-PCR analysis." Brain Res Brain Res Protoc **15**(2): 79-91.

Titelbaum, D. S., A. Degenhardt and R. P. Kinkel (2005). "Anti-tumor necrosis factor alpha-associated multiple sclerosis." AJNR Am J Neuroradiol **26**(6): 1548-1550.

Tkachenko, E., J. M. Rhodes and M. Simons (2005). "Syndecans: new kids on the signaling block." Circ Res **96**(5): 488-500.

Trapp, B. D. and K. A. Nave (2008). "Multiple sclerosis: an immune or neurodegenerative disorder?" Annu Rev Neurosci **31**: 247-269.

Tumova, S., B. A. Hatch, D. J. Law and K. J. Bame (1999). "Basic fibroblast growth factor does not prevent heparan sulphate proteoglycan catabolism in intact cells, but it alters the distribution of the glycosaminoglycan degradation products." Biochem J **337 (Pt 3)**: 471-481.

van Horssen, J., L. Bo, C. D. Dijkstra and H. E. de Vries (2006). "Extensive extracellular matrix depositions in active multiple sclerosis lesions." Neurobiol Dis **24**(3): 484-491.

van Horssen, J., C. D. Dijkstra and H. E. de Vries (2007). "The extracellular matrix in multiple sclerosis pathology." J Neurochem **103**(4): 1293-1301.

Van Vactor, D., D. P. Wall and K. G. Johnson (2006). "Heparan sulfate proteoglycans and the emergence of neuronal connectivity." Curr Opin Neurobiol **16**(1): 40-51.

Veugelers, M., B. De Cat, H. Ceulemans, A. M. Bruystens, C. Coomans, J. Durr, J. Vermeesch, P. Marynen and G. David (1999). "Glypican-6, a new member of the glypican family of cell surface heparan sulfate proteoglycans." J Biol Chem **274**(38): 26968-26977.

Willer, C. J., D. A. Dyment, A. D. Sadovnick, P. M. Rothwell, T. J. Murray, G. C. Ebers and G. Canadian Collaborative Study (2005). "Timing of birth and risk of multiple sclerosis: population based study." BMJ **330**(7483): 120.

Xue, H. H., P. E. Kovanen, C. A. Pise-Masison, M. Berg, M. F. Radovich, J. N. Brady and W. J. Leonard (2002). "IL-2 negatively regulates IL-7 receptor alpha chain expression in activated T lymphocytes." Proc Natl Acad Sci U S A **99**(21): 13759-13764.

Zuvich, R. L., J. L. McCauley, M. A. Pericak-Vance and J. L. Haines (2009). "Genetics and pathogenesis of multiple sclerosis." Semin Immunol **21**(6): 328-333.

Tables

Table 1. Population Demographics

	Total	Age (years)	Males	Age (years)	Females	Age (years)
Controls	194	19-96	43	29-70	151	19-96
Total Cases	205	18-77	45	24-77	160	18-76
Relapsing remitting (RR)	100	18-73	15	24-64	85	18-73
Secondary Progressive (SP)	51	37-73	11	37-70	40	40-73
Primary Progressive (PP)	54	24-77	19	28-77	35	24-76

Table 2. Assay details and SNP information including RFLP fragment sizes where appropriate

SNP number	Gene	Forward Primers	Reverse Primers	Chr	Chr Position	Amplicon Length (bp)	Variation	T_a (°C)	RFLP Fragment sizes (bp)	Accession Number	Assay Type
rs11546829	*EXT1*	5′ ACAGCCCCTTCCTTACCTGT 3'	5′ GGAAGTAAGGTCAGCCAAACC 3'	8	118847782	397	G/A	51	115, 281	NT_008046.16	RFLP
rs2623047	*SULF1*	5′ GGGATGCACAGAAACCCTAA 3'	5′ TGTGGCAAACAGTGAAGAGC 3'	8	70378496	291	C/T	57	212, 78	NT_008183.19	RFLP
rs1131351	*SDC1*	5′ TGCTGTACCGCATGAAGAAG 3'	5′ GCTGTGGTGGAAAGGTCCTA 3'	2	20402380	354	C/G	62	259, 94	NT_015926.15	RFLP
rs7333912	*GPC5*	5′ GGAAACATAACAAAGTTTGCAATC 3′	5′ TGGGGAGGGATAGGAAGATAAA 3′	13	91874131	120	C/G	49	N/A	NT_009952.14	HRM
rs10492503	*GPC5*	5' CTTCAATACTCTTGCTTGAATCGT 3'	5' CCGTAATTTGTGAGATATACCTTC 3'	13	92885097	115	A/T	58	N/A	NT_009952.14	HRM
rs9523787	*GPC5*	5′ TTCCTAGTTGATTGTTGAAGAGA 3′	5′ TGTAACCTTGATTTTCTTTCTAGT 3′	13	93363760	105	G/T	49	N/A	NT_009952.14	HRM
rs17267815	*GPC6*	5' ATGAGAGGGCTTCCATATAATCAT 3'	5' GGCAACAGTTTTGGAAGAAACA 3'	13	94153058	129	A/G	58	N/A	NT_009952.14	HRM
rs9524260	*GPC6*	5′ GACAGCCAGTGAATGTAGATAGGA 3′ Sequencing Primer	5′ Biotin-CAAATAACAGGAAGCTCAG 3′ 5' CAAATAACAGGAAGCTCAG 3'	13	94513790	105	G/A	56	N/A	NT_009952.14	Pyro

Table 3. Genotype and allele frequencies of EXT-1 SNP (rs11546829) within the case-control MS population which is further subdivided into disease states. Corrected p-values using the Benjimini-Hochberg (P_{BH}) and Bonferroni (P_{Bon}) methods are presented below the uncorrected p-value.

	EXT1-829	rs11546829							
Polymorphism Group	Genotypes					Alleles			
	GG (%)	GA (%)	AA (%)	*P* (P_{BH}, P_{Bon})	HWE	G (%)	A (%)	*P*	OR (95% CI)
MS Total Cases (n=176)	80 (45.5)	78 (44.3)	18 (10.2)	0.45 (0.72, 1)	0.87	238 (67.6)	114 (32.4)	0.75	0.95
PP Case (n=49)	22 (44.9)	22 (44.9)	5 (10.2)	0.67		66 (67.3)	32 (32.7)	0.87	0.96
SP Case (n=43)	21 (48.8)	19 (44.2)	3 (7)	0.45		61 (70.9)	25 (29.1)	0.44	0.81
RR Case (n=84)	37 (44)	37 (44)	10 (12)	0.72		111 (66.1)	57 (51.4)	0.94	1.02
Control (n=134)	63 (47)	52 (38.9)	19 (14.2)	-	0.13	178 (66.4)	90 (33.6)	-	-
HapMap CEU (%)	58.3	36.7	5			76.7	23.3		

Table 4. Genotype and allele frequencies in the case-control MS population for the SULF-1 SNP (rs262347) further subdivided into disease states. Corrected p-values using the Benjimini-Hochberg (P_{BH}) and Bonferroni (P_{Bon}) methods are presented below the uncorrected p-value.

	SULF1	rs2623047							
Polymorphism Group	Genotypes					Alleles			
	CC (%)	CT (%)	TT (%)	*P* (P_{BH}, P_{Bon})	HWE	C (%)	T (%)	*P*	OR (95% CI)
MS Total Cases (n=190)	21 (11.1)	84 (44.2)	85 (44.7)	0.94 (0.94, 1)	0.97	126 (33.2)	254 (66.8)	0.94	1.01
PP Case (n=50)	7 (14)	20 (40)	23 (46)	0.68		34 (34)	66 (66)	0.92	0.97
SP Case (n=46)	8 (17.4)	18 (39.1)	20 (43.5)	0.40		34 (37)	58 (63)	0.53	0.86
RR Case (n=94)	6 (6.4)	46 (48.9)	42 (44.7)	0.53		58 (30.9)	130 (69.1)	0.54	1.13
Control (n=172)	18 (10.5)	79 (45.9)	75 (43.6)	-	0.68	115 (33.4)	229 (66.6)	-	-
HapMap CEU (%)	15.9	51.3	32.7			41.6	58.4		

Table 5. Results by disease state for SDC1 SNP (rs1131351) and of the MS case-control population. Corrected p-values using the Benjimini-Hochberg (P_{BH}) and Bonferroni (P_{Bon}) methods are presented below the uncorrected p-value.

	SDC 1	rs113151							
Polymorphism	Genotypes					Alleles			
Group	GG (%)	GC (%)	CC (%)	P	HWE	G (%)	C (%)	P	OR (95% CI)
				(P_{BH}, P_{Bon})					
MS Total Cases (n=160)	31 (19.4)	83 (51.9)	46 (28.8)	**0.02**	0.55	145 (45.3)	175 (54.7)	**0.004**	1.59
				(0.08, 0.16)					
PP Case (n=45)	8 (17.8)	24 (53.3)	13 (28.9)	0.11		40 (44.4)	50 (55.6)	**0.04**	1.65
SP Case (n=39)	8 (20.5)	19 (48.7)	12 (30.8)	0.15		35 (44.9)	43 (55.1)	0.06	1.62
RR Case (n=76)	15 (19.7)	40 (52.6)	21 (27.6)	0.09		70 (46.1)	82 (53.9)	**0.03**	1.55
Male (n=31)	7 (22.6)	17 (54.8)	7 (22.6)	0.58		31 (50)	31 (50)	0.32	1.32
Female (n=129)	24 (18.6)	66 (51.2)	39 (30.2)	**0.01**		114 (44.2)	144 (58.8)	**0.003**	1.67
Total Control (n=145)	46 (31.7)	73 (50.3)	26 (17.9)	-	0.75	165 (56.9)	125 (43.1)	-	
HapMap CEU (%)	50	31	19			65.5	34.5		

Table 6. Female results by disease state for SDC1 SNP (rs1131351)

	SDC1	rs1131351						
Polymorphism	Genotypes				Alleles			
Group	GG (%)	GC (%)	CC (%)	*P*	G (%)	C (%)	*P*	OR (95% CI)
Female MS Case								
PP (n=31)	3 (9.68)	17 (54.8)	11 (35.5)	**0.02**	23 (37.1)	39 (62.9)	**0.005**	2.24
SP (n=28)	7 (25)	13 (46.4)	8 (28.6)	0.41	27 (48.2)	29 (51.8)	0.23	1.42
RR (n=70)	14 (20)	36 (51.4)	20 (28.6)	0.09	64 (45.7)	76 (54.3)	**0.03**	1.57
Total Control (n=145)	46 (31.7)	73 (50.3)	26 (17.9)	-	165 (56.9)	125 (43.1)	-	-
HapMap CEU (%)	50	31	19		65.5	34.5		

Table 7. Results for GPC5, rs7333912 Corrected p-values using the Benjimini-Hochberg (P_{BH}) and Bonferroni (P_{Bon}) methods are presented below the uncorrected p-value.

	GPC5	rs7333912							
Polymorphism	Genotypes					Alleles			
Group	CC (%)	GC (%)	GG (%)	*P*	HWE	C (%)	G (%)	*P*	OR (95% CI)
				(P_{BH}, P_{Bon})					
MS Total Cases (n=205)	195 (95.1)	10 (4.9)		0.768	0.72	400 (97.6)	10 (2.4)	0.771	1.15
				(0.878, 1)					
PP Case (n=54)	52 (96.3)	2 (3.7)				106 (98.1)	2 (1.9)	0.859	0.87
SP Case (n=51)	47 (92.2)	4 (7.8)				98 (95.1)	4 (3.9)	0.189	2.11
RR Case (n=100)	96 (96.0)	4 (4.0)				196 (98.0)	4 (2.0)	0.667	0.75
Control (n=188)	180 (95.7)	8 (4.3)			0.766	368 (97.9)	8 (2.1)		
HapMap CEU (%)	99.1	0.9				99.6	0.4		

Table 8. Results for GPC5, rs10492503. Corrected p-values using the Benjimini-Hochberg (P_{BH}) and Bonferroni (P_{Bon}) methods are presented below the uncorrected p-value.

	GPC5	rs10492503							
Polymorphism	Genotypes					Alleles			
Group	AA (%)	AT (%)	TT (%)	*P* (P_{BH}, P_{Bon})	HWE	A (%)	T (%)	*P*	OR (95% CI)
MS Total Cases (n=204)	67 (32.8)	98 (48.1)	39 (19.1)	**0.016** (0.08, 0.128)	0.767	232 (56.8)	176 (43.2)	**0.0079**	1.50
PP Case (n=53)	24 (45.3)	24 (45.3)	5 (9.4)	0.492		72 (67.9)	34 (32.1)	0.781	0.94
SP Case (n=51)	13 (25.5)	31 (60.8)	7 (13.7)	**0.0098**		57 (55.9)	45 (44.1)	0.0519	1.56
RR Case (n=100)	30 (30)	43 (43)	27 (27)	**0.0067**		103 (52.5)	97 (48.5)	**0.0006**	1.87
Male (n=44)	14 (31.8)	21 (47.7)	9 (20.5)	0.560		49 (55.7)	39 (44.3)	0.299	1.42
Female (n=160)	53 (33.1)	77 (48.1)	30 (18.8)	**0.0286**		183 (57.2)	137 (42.8)	**0.0148**	1.52
Control (n=164)	78 (47.6)	62 (37.8)	24 (14.6)		0.052	218 (66.5)	110 (33.5)		
HapMap CEU (%)	38.3	51.7	10			64.2	35.8		

Table 9. Female results by disease state for GPC5, rs10492503. Significance for the PP case subgroup is suggestive only as cell counts fell below the minimum required for Chi-squared testing (n<5).

	GPC5	rs10492503						
Polymorphism	Genotypes				Alleles			
Group	AA (%)	AT (%)	TT (%)	*P*	A (%)	T (%)	*P*	OR (95% CI)
Female MS Case								
PP (n=35)	17 (48.6)	16 (45.7)	2 (5.7)	0.3358	50 (71.4)	20 (28.6)	0.4847	0.81
SP (n=40)	9 (22.5)	25 (62.5)	6 (15.0)	**0.0087**	43 (53.8)	37 (46.2)	**0.030**	1.75
RR (n=85)	27 (31.8)	36 (42.4)	22 (25.9)	**0.0245**	90 (52.9)	80 (47.1)	**0.0032**	1.81
Total Control (n=132)	64 (48.5)	49 (37.1)	19 (14.4)	-	177 (67.1)	87 (32.9)	-	-
HapMap CEU (%)	38.3	51.7	10		64.2	35.8		

Table 10. Results for GPC5, rs9523787. Corrected p-values using the Benjimini-Hochberg (P_{BH}) and Bonferroni (P_{Bon}) methods are presented below the uncorrected p-value. Significance measures are suggestive only as cell counts fell below the minimum required to perform Chi-square analysis in the disease subgroups (n<5).

	GPC5	rs9523787							
Polymorphism	Genotypes					Alleles			
Group	GG (%)	GT (%)	TT (%)	*P* (P_{BH}, P_{Bon})	HWE	G (%)	T (%)	*P*	OR (95% CI)
MS Total Cases (n=205)	146 (71.2)	54 (26.3)	5 (2.5)	0.609 (0.812,1)	0.998	346 (84.4)	64 (15.6)	0.464	0.87
PP Case (n=54)	37 (68.5)	17 (31.5)	0 (0)	0.558		91 (84.3)	17 (15.7)	0.660	0.88
SP Case (n=57)	40 (78.4)	9 (17.7)	2 (3.9)	0.153		89 (87.3)	13 (12.7)	0.246	0.69
RR Case (n=94)	69 (69.0)	28 (28.0)	3 (3.0)	0.811		166 (83.0)	34 (17.0)	0.868	0.96
Control (n=188)	126 (67.0)	58 (30.9)	4 (2.1)		0.366	310 (82.4)	66 (17.6)		
HapMap CEU (%)	64.6	27.4	8			78.3	21.7		

Table 11. Results for GPC6, rs17267815. Corrected p-values using the Benjimini-Hochberg (P_{BH}) and Bonferroni (P_{Bon}) methods are presented below the uncorrected p-value.

	GPC6	rs17267815							
Polymorphism	Genotypes					Alleles			
Group	AA (%)	AG (%)	GG (%)	*P*	HWE	A (%)	G (%)	*P*	OR (95% CI)
				(P_{BH}, P_{Bon})					
MS Total Cases (n=205)	46 (22.4)	105 (51.2)	54 (26.4)	0.0797	0.71	197 (48.1)	213 (51.9)	0.118	0.79
				(0.213, 0.638)					
PP Case (n=54)	10 (18.5)	26 (48.2)	18 (33.3)	0.691		46 (42.6)	62 (57.4)	0.925	0.98
SP Case (n=51)	8 (15.7)	29 (56.9)	14 (27.4)	0.161		45 (51.7)	42 (48.3)	0.719	0.92
RR Case (n=100)	28 (28.0)	50 (50.0)	22 (22.0)	**0.039**		106 (51.7)	94 (48.3)	**0.017**	0.64
Male (n=45)	14 (31.1)	20 (44.4)	11 (24.4)	0.1436		48 (53.3)	42 (46.7)	**0.0386**	0.51
Female (n=160)	32 (20.0)	85 (53.1)	43 (26.9)	0.1154		149 (46.6)	171 (53.4)	0.5095	0.89
Control (n=145)	31 (21.4)	60 (41.4)	54 (37.2)		0.069	205 (54.5)	171 (45.5)		
HapMap CEU (%)	26.5	54	19.5			53.5	46.5		

Supplementary Table 1. Male results by disease state for GPC6, rs17267815

	GPC6	rs17267815						
Polymorphism	Genotypes				Alleles			
Group	AA (%)	AG (%)	GG (%)	*P*	A(%)	G (%)	*P*	OR (95% CI)
Male MS Case								
PP (n=19)	3 (15.8)	9 (47.4)	7 (36.8)	0.9533	15 (39.5)	23 (60.5)	0.7826	0.89
SP (n=11)	3 (27.3)	6 (54.6)	2 (18.2)	0.3383	12 (54.6)	10 (45.4)	0.1407	0.48
RR (n=15)	8 (53.3)	5 (33.3)	2 (13.3)	**0.0134**	21 (70)	9 (30)	**0.0024**	0.25
Total Control (n=34)	5 (14.7)	15 (44.1)	14 (41.2)	-	25 (36.8)	43 (63.2)	-	-
HapMap CEU (%)	26.5	54	19.5		53.5	46.5		

Table 12. Results for GPC6, rs9524260. Corrected p-values using the Benjimini-Hochberg (P_{BH}) and Bonferroni (P_{Bon}) methods are presented below the uncorrected p-value.

	GPC6	rs9524260							
Polymorphism	Genotypes					Alleles			
Group	GG (%)	GA (%)	AA (%)	*P*	HWE	G (%)	A (%)	*P*	OR (95% CI)
				(P_{BH}, P_{Bon})					
MS Total Cases (n=197)	76 (38.6)	90 (45.7)	31 (15.7)	0.236	0.613	242 (61.4)	152 (38.6)	0.974	1.00
				(0.472, 1)					
PP Case (n=53)	22 (41.5)	21 (39.6)	10 (18.9)	0.146		65 (61.3)	41 (38.7)	0.967	1.01
SP Case (n=50)	16 (32.0)	24 (48.0)	10 (20.0)	0.296		56 (56.0)	44 (44.0)	0.316	1.26
RR Case (n=94)	38 (40.4)	45 (47.9)	11 (11.7)	0.607		121 (64.4)	67 (35.6)	0.516	0.89
Control (n=182)	63 (34.6)	98 (53.8)	21 (11.6)		0.064	224 (61.5)	140 (38.5)		
HapMap CEU (%)	36.3	54.9	8.8			63.7	36.3		

Table 13. Comparison of significance obtained in this study compared to previous GWAS. P-values from GWAS presented as from original paper. P_{un} = uncorrected P-value; P_C = Corrected P-value. Baranzini et al presented their significance as Adjusted Log P-values.

GENE	SNP	GENOTYPE (%)			ALLELE (%)		Current Study			GWAS Significance		
		Hom (%)	Het (%)	Var (%)	Allele 1	Allele 2	P-value	Corrected B-H	Corrected Bonferroni	Cenit 2009 P_{un} (P_C)	Baranzini 2009 Adj Log P-value	Lorentzen 2010 P_{un}
GPC5	rs7333912	95.1	4.9	0	97.6	2.4	0.76	0.878	1			0.02
GPC5	rs10492503	32.8	48.1	19.1	56.8	43.2	**0.016**	0.08	0.128	0.016 (0.096)		
GPC5	rs9523787	71.2	26.3	2.5	84.4	15.6	0.069	0.812	1			0.0002
GPC5	rs9523762	DID NOT FOLLOW HARDY-WEINBURG EQUILIBRIUM									5.155	
GPC6	rs17267815	22.4	51.2	26.4	48.1	51.9	0.0797	0.213	0.638			0.03
GPC6	rs9524260	38.6	45.7	15.7	61.4	38.6	0.236	0.472	1			0.10

Figures

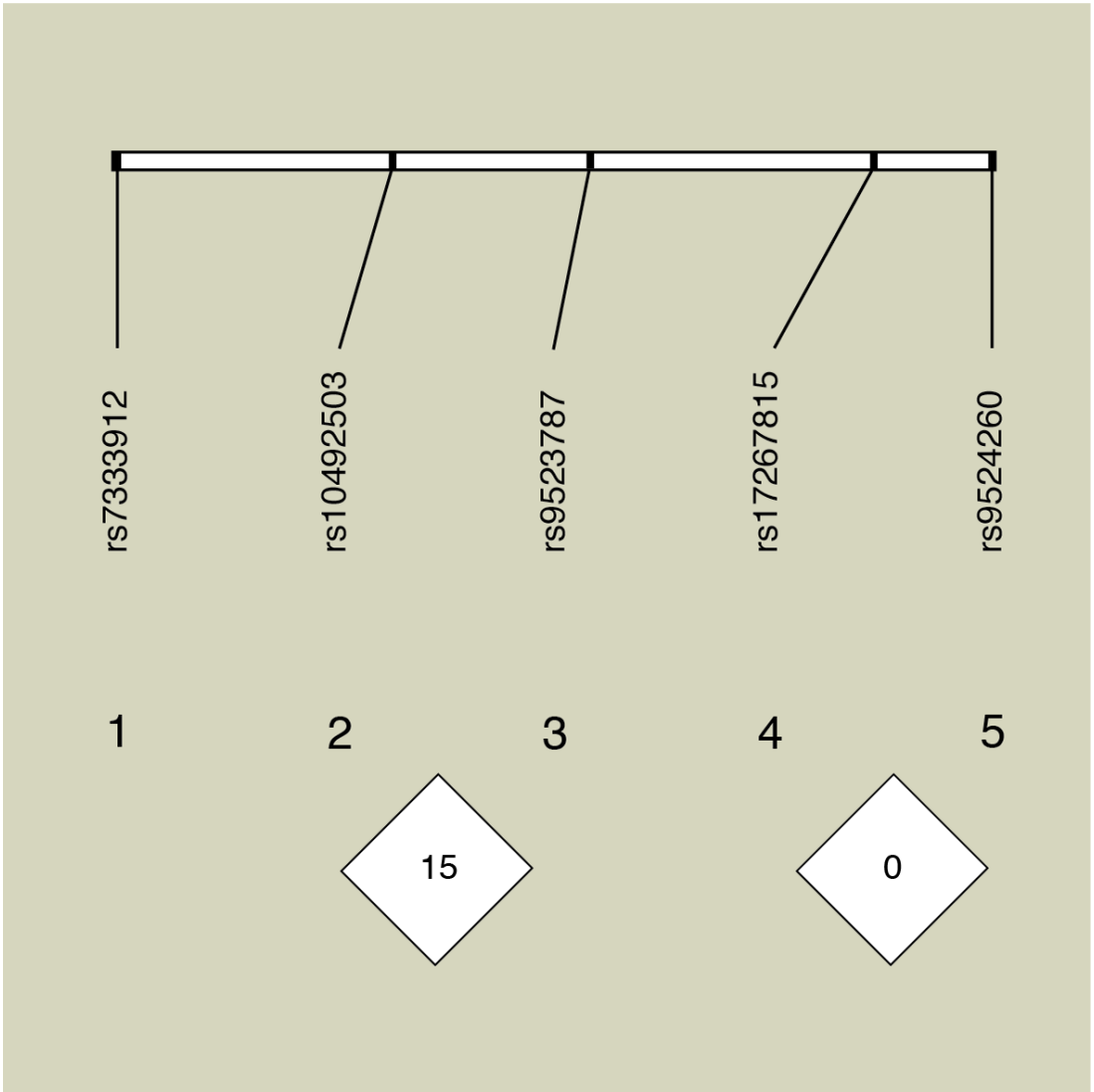


Figure 1

LD Plot from GPC5 / GPC6 haplotype analysis. The figure shows there is no LD between these SNPs in an Australian Caucasian MS population. This analysis was unable to replicate the positive associations and LD found in previous studies.

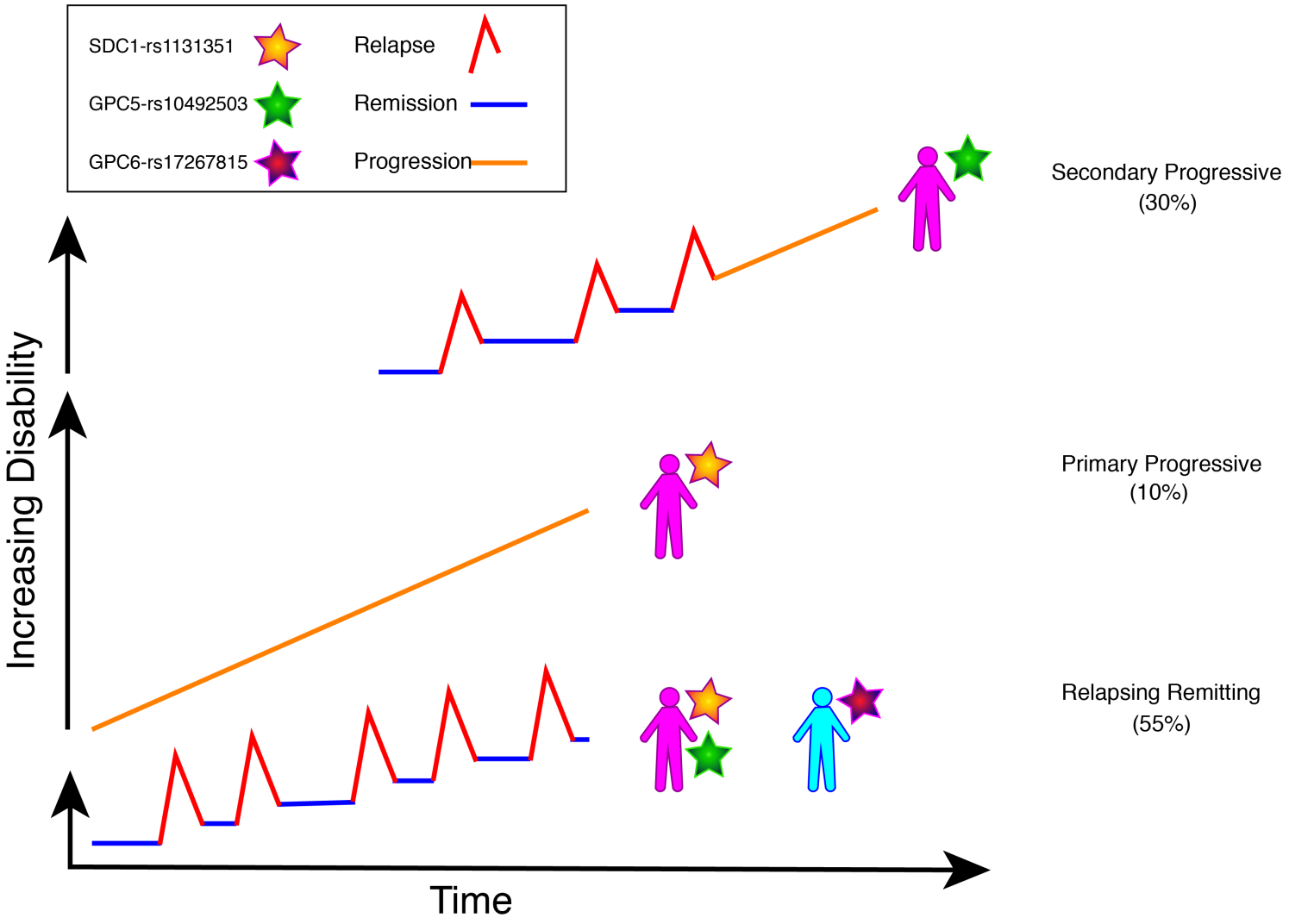


Figure 2

Schematic of disease progression highlighting involvement of HSPG core protein SNPs. Significant associations between specific HSPG core protein SNPs and disease subtypes are represented by coloured stars as can be seen in the legend. SDC1: yellow; GPC5: green; GPC6: purple. These SNPs are significantly associated with population stratification by gender as represented by coloured people outlines. Female: pink; Male: blue.