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Farmacogenética da esquizofrenia

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Lista de abreviaturas

AC	Adenilato ciclase
AAAD	Descarboxilase de L-aminoácidos aromáticos
AMPA	Alfa-amino-3-hidróxido-5-metil-isoxazole-propionato
AMPc	Adenosina monofosfato cíclico
BDNF	Fator neurotrófico derivado do cérebro
cDNA	Ácido desoxiribonucleico complementar
Cdk-5	Quinase dependente de ciclina 5
CK	Caseína quinase
COMT	Catecol-O-metil-transferase
CREB	Elemento ligante responsivo a AMPc
Da	Dalton
DARPP-32	Fosfoproteína regulada por dopamina e AMPc de 32 KDa
DAT	Transportador de dopamina
DNA	Ácido desoxiribonucleico
dNTP	Desoxiribonucleotídeo trifosfato
EEC	Estimulação eletroconvulsiva
ECT	Eletroconvulsoterapia
FGF-2	Fator de crescimento para fibroblasto
GABA	Ácido γ-aminobutírico
GRK2	Proteína quinase 2 acoplada à proteína G
L-DOPA	3,4-dihidroxifenilalanina
LTD	Depressão de longa duração
LTP	Potencial de longa duração
MAO	Monoamino oxidase
NMDA	N-metil-D-aspartato
NCS-1	Sensora de cálcio neuronal 1
NGF	Fator de crescimento neuronal
PAGE	Eletroforese em gel de poliacrilamida

PBS	Tampão fosfato salino
PKA	Proteína quinase A
PKG	Proteína quinase G
PP	Proteína fosfatase
RNA	Ácido ribonucleico
SDS	Dodecil sulfato de sódio
SNC	Sistema nervoso central
Ser	Serina
TDAH	Transtorno de déficit de atenção e hiperatividade
TH	Tirosina hidroxilase
Thr	Treonina
VMAT	Transportador vesicular de monoaminas

Lista de figuras

Resumo

Conhecidos há aproximadamente 2500 anos, os transtornos do humor continuam a dominar o interesse da saúde pública. Cerca de 5% dos pacientes depressivos não respondem a qualquer medida farmacológica e/ou psicoterápica. Para esses pacientes, a eletroconvulsoterapia (ECT) constitui uma importante oportunidade de melhora.

Indução de convulsões na forma de ECT tem sido usada no tratamento de desordens psiquiátricas por mais de 60 anos. As principais indicações diagnósticas incluem depressão, mania, catatonia e esquizofrenia. Devido às dificuldades na identificação dos mecanismos de ação da ECT, têm-se usado a estimulação eletroconvulsiva (EEC) aplicada experimentalmente a animais com o intuito de obter dados que contribuam para explicar alguns efeitos terapêuticos da ECT. Há relatados de alteração nos níveis das proteínas DARPP-32 (fosfoproteína regulada por AMPc e dopamina) e NCS-1 (sensora neuronal de cálcio 1) em pacientes com transtornos neuropsiquiátricos.

Neste trabalho foram avaliados os níveis de expressão das proteínas DARPP-32 e NCS-1 em quatro regiões cerebrais (*striatum*, córtex, hipocampo e cerebelo) de ratos submetidos ao choque eletroconvulsivo agudo e crônico. A estimulação eletroconvulsiva aguda gerou aumento na expressão de DARPP-32 no córtex. É interessante notar que nessa área as alterações foram observadas logo após a realização do estímulo e após 24 horas sendo essa sustentada até às 48 horas. Nas outras áres avaliadas (*striatum*, hipocampo e cerebelo) não foram observadas alterações significativas. Tais achados corroboram a ausência de eficácia dessa terapêutica de modo agudo usualmente.

A estimulação crônica provocou alterações significativas em todas as áreas estudadas, o que está de acordo com a utilização de repetidas sessões desta técnica na clínica. A estimulação eletroconvulsiva aguda gerou somente alterações pontuais nos níveis de NCS-1 no córtex (diminuição em 48 horas), hipocampo (diminuição em 03 horas) e cerebelo (aumento em 3 horas). A estimulação crônica gerou modificações importantes no *striatum* e córtex, cerebelo. No *striatum*, aumento é notado logo após do último estímulo e a partir de 03 horas até às 24 horas. No córtex e cerebelo, o aumento é evidente de 12 horas até às 48 horas. Tais dados, assim como os obtidos para DARPP-32, mostram uma maior eficiência da terapia crônica em relação à aguda e uma dinâmica temporal que favorece a aplicação da técnica num intervalo de 2-3 dias (tempo pelo qual manteve-se os níveis elevados das proteínas).

1. Introdução

1.1 – Esquizofrenia

A esquizofrenia é um transtorno psicótico maior (ou um grupo de transtornos) que usualmente aparece na fase mais tardia da adolescência ou no início da idade adulta, sendo esta uma doença relativamente comum. Sua prevalência ao longo da vida é de 0,5-1% na população geral, mas essa estimativa pode variar de acordo com a metodologia utilizada nos diferentes estudos (Freedman, 2003). Os estudos epidemiológicos realizados no Brasil revelam estimativas de incidência e prevalência compatíveis com as observadas em outros países. Dados do Ministério de Saúde indicam que aproximadamente 1% da população brasileira já sofreu um episódio psicótico e estima-se que aproximadamente 80.000 pessoas são acometidas a cada ano. As taxas médias para homens e mulheres são similares, mas a idade média de início é cerca de cinco anos maior para as mulheres do que para os homens, sendo estes mais frequentemente afetados por sintomas negativos do que mulheres (Häfner, 2003). As mulheres tendem a apresentar um curso mais brando da esquizofrenia e, portanto, um melhor prognóstico e uma melhor possibilidade de adaptação social (Austin, 2005).

A importância da pesquisa sobre esquizofrenia é diretamente proporcional a sua importância social e econômica, em especial pelo fato que os indivíduos são geralmente acometidos no auge do seu potencial produtivo, entre 16 e 30 anos de idade, gerando uma sobrecarga para os pacientes e seus familiares. Na maioria dos casos, há prejuízo das funções ocupacionais ou sociais, caracterizado por afastamento social, perda de interesse ou capacidade de agir na escola ou no trabalho, mudança nos hábitos de higiene pessoal ou comportamento incomum (Loebel e cols, 1992; Häfner, 2003). Pacientes sofrem de um estresse considerável, tem sua qualidade de vida diminuída e enfrentam incapacidades prolongadas que podem impor efeitos negativos em seus empregos, nos orçamentos pessoal ou familiar, no relacionamento afetivo e na satisfação com a vida. Após passar pela fase aguda, o transtorno pode persistir, e períodos de remissão se alternam com os períodos de exacerbação (Austin, 2005).

Além de comprometer pacientes e familiares, há ainda um grande custo para toda a sociedade (tratamento e custos indiretos, como mortalidade e redução da produtividade). Após um segundo episódio psicótico, por exemplo, a taxa de desemprego entre pacientes com esquizofrenia é superior a 65%, contribuindo para o alto custo indireto da doença (Guest e Cookson, 1999). No Brasil, a esquizofrenia ocupa 30% dos leitos psiquiátricos hospitalares, ou

cerca de 100 mil leitos-dia. Ocupa ainda o segundo lugar das primeiras consultas psiquiátricas ambulatoriais (14%) e o quinto lugar na manutenção de auxílio-doença. Os gastos anuais do Sistema Único de Saúde com a internação e tratamento de 131 mil pacientes com esquizofrenia consomem quantias elevadas de recursos (Javitt e Coyle, 2004). Na Inglaterra, cerca de 6% dos custos com pacientes internados no Serviço Nacional de Saúde são relativos à esquizofrenia (Knapp, 1997), sendo esta responsável por 2,5% dos gastos com atendimento anual de saúde nos Estados Unidos (Rupp e Keith, 1993).

A esquizofrenia apresenta sintomas diferentes em múltiplos domínios, de forma muito heterogênea em diferentes indivíduos e também variabilidade nos mesmos indivíduos ao longo do tempo. Pela observação sistemática da psicopatologia, fenômenos positivos e negativos podem ser separados (Andreasen, 1982; Crow, 1985). Os sintomas positivos, de maneira abrangente, incluem delírios e ideação delirante, alucinações, distúrbios das associações, sintomas catatônicos, agitação, vivências de influência externa e desconfiança. Os sintomas negativos referem-se ao estreitamento e à redução das expressões emocionais, com diminuição da produtividade do pensamento e da fala, retraimento social e diminuição dos comportamentos direcionados a metas. Como uma terceira categoria, os sintomas desorganizados incluem desorganização do pensamento e do comportamento associado ao comprometimento da atenção.

A avaliação neuropsicológica longitudinal mostrou que os pacientes com esquizofrenia têm disfunção cognitiva considerável nos primeiros cinco anos da doença. Após esse período, há poucas evidências de deterioração (Hoff e cols, 1999). Aproximadamente metade dos pacientes com esquizofrenia tratados em serviços convencionais irão recidivar e necessitarão de readmissão nos primeiros dois anos, chegando a até 80% de recidivas num período de cinco anos (Robinson e cols, 1999); cerca de 10% a 25% não terão admissões posteriores (Fenton e McGlashan, 1987; Hegarty e cols, 1994). Anteriormente às recidivas, sinais de aviso freqüentemente aparecem, os quais usualmente consistem em sintomas não-psicóticos seguidos por distúrbios emocionais e sintomas psicóticos leves ao longo de um período de 4 a 12 semanas (Birchwood e cols, 1999; Gaebel e cols, 1993).

Fatores preditivos associados em geral à melhor evolução são: início mais tardio em relação às faixas etárias usuais, gênero feminino, indivíduos casados, personalidade pré-mórbida sociável, bom ajuste e bom funcionamento pré-mórbido, quociente intelectual mais elevado, presença de um desencadeante na esfera emocional quando do início, início agudo, ausência de

complicações perinatais, sintomas predominantemente afetivos ou sintomas positivos e ausência de desorganização ou sintomas negativos quando do início, menor número de episódios prévios, padrão por fases de episódios e remissões, bem como ausência de histórico familiar de esquizofrenia (Hegarty e cols, 1994; Davidson e McGlashan, 1997; Bottlender e cols, 2000, 2002 e 2003; Häfner, 2003).

Outros transtornos mentais comórbidos e condições médicas gerais são freqüentemente encontrados em pacientes com esquizofrenia o que colabora para uma expectativa de vida reduzida em relação à população em geral. A elevada taxa de mortalidade observada em pacientes esquizofrênicos deve-se, principalmente, ao elevado risco de suicídio (de 4 a 15% dos pacientes cometem suicídio); aos distúrbios cardiovasculares; às doenças respiratórias e infecciosas; e às injúrias traumáticas (Brown e cols, 2000). Uma das condições comórbidas mais freqüentes é o abuso de drogas, que ocorre em 15 a 71% dos pacientes com esquizofrenia (Soyka e cols, 1993; Kovasznay e cols, 1997; Bersani e cols, 2002). Fatores que influenciam o risco de abuso de substâncias estão associados ao meio ambiente social (pregresso e atual) e à personalidade pré-mórbida (Arndt e cols, 1992). Condições comórbidas podem piorar o curso e complicar o tratamento (Linszen e cols, 1994).

1.2 – Etiologia da esquizofrenia

A esquizofrenia é uma doença de etiologia complexa. Diversos grupos de pesquisa procuraram determinar o papel de variáveis biológicas específicas, tais como os fatores genéticos e bioquímicos e alterações sutis na morfologia cerebral, mas não se encontrou ainda uma alteração suficiente para explicar a etiopatogênese da doença. As idéias para tais estudos sobre a patofisiologia da esquizofrenia basearam-se em estudos farmacológicos por muitos anos. A clássica hipótese dopaminérgica suportava a presença de estado hiperdopaminérgico decorrente da potência das drogas típicas utilizadas (Seeman e Lee, 1975), gerando uma modulação diferenciada da atividade dopaminérgica devido à produção aumentada desse neurotransmissor, ou uma hipersensibilidade dos receptores de dopamina no sistema mesolímbico resultando em hiperexcitabilidade e aparecimento de sintomas positivos, e de um estado hipodopaminérgico em regiões cerebrais frontais, associado aos sintomas negativos (Sedvall e Farde, 1995). Essa hipótese tem como suporte o tratamento bem-sucedido dos sintomas psicóticos por agentes bloqueadores dos receptores de dopamina do tipo 2 (DRD2) no sistema mesolímbico.

Dados mostram que há um aumento da transmissão dopaminérgica no gânglio basal associado com um quadro de psicose (Abi-Dargham e cols, 2000) e uma alteração na resposta dopaminérgica no córtex pré-frontal em pacientes apresentando disfunções cognitivas crônicas (Weinberger e cols, 2001). Estudos psicofarmacológicos demostraram que, além da dopamina, outros neurotransmissores (como a serotonina e o glutamato) parecem estar envolvidos na fisiopatologia da esquizofrenia. (Meltzer e cols, 1989; Javitt e Zukin, 1991). No entanto, como todos esses sistemas interagem, essas hipóteses não são mutualmente excludentes, e, ao menos, a modulação do sistema dopaminérgico pode ser uma ação secundária de uma alteração na sinalização glutamatérgica cortical (Coyle, 2006).

Algumas das evidências para o papel do glutamato são oriundas da farmacologia, tais como o fato de fenilciclidina e a cetamina, antagonistas do receptor de glutamato N-metil-D-aspartato (NMDA), poderem causar anormalidades cognivitas e psicóticas; e pacientes com esquizofrenia parecem ser especialmente sensíveis aos efeitos psicomiméticos dessas drogas (Ross e cols, 2006). De maneira interessante, ensaios clínicos demonstraram que agentes que modulam o receptor de NMDA (glicina, D-serina, D-cicloserina e D-alanina) melhoram, em especial, sintomas negativos quando combinados com antipsicóticos (Heresco-Levy e cols, 2002;

Lane e cols, 2005; Tsai e Gleeson; 2005). Esses dados revelam que a hipofunção do receptor NMDA, que está relacionada de maneira crítica com a função de interneurônios produtores de ácido-gamaaminobutírico (GABA), poderia contribuir para a patofisiologia da esquizofrenia (Ross e cols, 2006).

O potencial papel do GABA, principal neurotransmissor inibitório no sistema nervoso de mamíferos e que é sintetizado a partir do glutamato, na patogênese da esquizofrenia é resultante, em sua maior parte, de estudos neuropatológicos (Lewis e cols, 2005). Um subtipo particular de interneurônios de GABA, conhecidos como *chandelier*, apresentam redução da imunomarcação para o transportador de GABA, possivelmente relacionada à redução da sinalização do fator neurotrófico derivado do cérebro (BDNF) ou hipofunção do receptor NMDA. Consistente com a possível redução do transporte de GABA, estudos imunocitoquímicos e de ligação demostraram maior atividade dos receptores de GABA do tipo A ($GABA_A$) nessa área. Entretanto, não é clara qual a extensão da relevância desses neurotrasmissores na etiopatogênese da esquizofrenia (Ross e cols, 2006).

Alguns resultados mostram relações entre alterações do neurodesenvolvimento e esquizofrenia, tal como a maior incidência de problemas motores e neuropsicológicos em crianças com maior chance de desenvolver esquizofrenia e aumento ventricular e redução do volume cortical nos pacientes com esquizofrenia (Lawrie e cols, 1999; Pantelis e cols, 2003). A redução de algumas estruturas cerebrais relatadas podem em princípio ser o resultado do neurodesenvolvimento anormal e/ou neurodegenereção. A existência de um mecanismo alterado durante o neurodesenvolvimento é suportado pela falha de se encontrar marcadores de processos neurodegenerativos (Harrison, 1999). Por sua vez, estudos neuropatológicos também falharam em estabelecer um diagnóstico claro e alguns dados são controversos. Apesar disso, há evidências apresentadas por mais de um estudo relatando: redução do tamanho neuronal, em especial no lobo temporal, córtex pré-frontal e dorso talâmico (Harrison, 1999). Estas alterações, junto com reduções vistas em marcadores sinápticos e dendríticos e anormalidades na substância branca (Akbarian e cols, 1996; Davis e cols, 2003), sugerem problemas na estrutura sináptica e na função, bem como na conectividade, dos neurônios (Arnold e cols, 2005).

Os fatores desencadeantes, independente de quais são, parecem atuar de forma mais importante durante o neurodesenvolvimento, durante os períodos pré e perinatal, do que somente uma simples manifestação imediatamente prévia ao surgimento dos sintomas (Murray e Lewis,

1987; Weinberg, 1995, Marenco e Weinberger, 2000). Os fatores que poderiam gerar tais distúrbios podem ser desde uma exposição à infecção por influenza, herpes vírus, citomegalovírus, vírus do pólio ou *Toxoplasma gondii* já no primeiro trimestre de gravidez ou outros fatores no segundo e terceiro trimestres de gestação (Brown e Susser, 2002). Entre esses fatores de risco pode-se apresentar: rubéola e infecções respiratórias; nascimentos em baixas classes sócio-econômicas; nascimentos na zona urbana; baixo peso; incompatibilidade de fator Rh; complicações durante o parto; e nascimento durante fim do inverno e início da primavera (Lewis e cols, 1987; Dohrenwend e cols, 1992; Marcelis e cols, 1999; Susser e cols, 1996; Torrey e cols, 1997; Cannon e cols, 2002; Kyle e Pichard, 2006; St Clair e cols, 2005). O quanto a doença vai se apresentar ou não pode ser o resultado da combinação entre fatores genéticos e ambientais. Segundo Gottesman e Bertelsen (1989) e Gottesman (1991) os componentes genéticos poderiam explicar apenas metade do risco de desenvolver esquizofrenia, e as complicações pré ou perinatais seriam responsáveis por aproximadamente 1% desse risco.

1.3 – Estudos genéticos em esquizofrenia

O balanço entre componentes genéticos e ambientais – estes podendo ser biológicos ou psicossociais – estão provavelmente presentes na etiologia da maioria dos transtornos mentais (Rutter e cols, 1999). Como foi enfatizado pelo *U.S. Department of Health e Human Services* no *Mental Health´s Report of the Surgeon General* em 1999:

> "*...dois pontos importantes sobre fatores biológicos devem ser mantidos em mente. O primeiro é que influências biológicas não são necessariamente sinônimas às de origem genética ou herdáveis. Anormalidades biológicas do sistema nervoso central que influenciam o comportamento, o pensamento ou os sentimentos podem ser causadas por trauma físico, infecções, desnutrição, ou exposição à toxinas, tais como contaminação do ambiente com chumbo. Essas anormalidades não são herdáveis. Transtornos mentais que têm maior probabilidade de ter componentes genéticos incluem autismo, transtorno bipolar, esquizofrenia e transtorno de deficit de atenção e hiperatividade (TDAH). Segundo, é errôneo assumir que fatores biológicos e ambientais são independentes um do outro, quando de fato eles interagem. Por exemplo, experiências traumáticas podem induzir alterações biológicas persistentes. Em contrapartida, crianças com um comportamento anormal com base biológica podem modificar seu ambiente...*".

O modelo apresentado por Dawson e Nuechterlein (1984) integra de forma interessante estes componentes, propondo que a vulnerabilidade resultará no desenvolvimento de sintomas quando estressores ambientais estiverem presentes e os mecanismos para lidar com eles falharem. Os fatores de vulnerabilidade, baseados em um componente biológico que inclui a predisposição genética, seriam capazes de interagir com fatores complexos físicos, ambientais e psicológicos de vulnerabilidade.

Algumas doenças seguem um padrão de herança mendeliana simples, tal como a doença de Huntington e fibrose cística. Essas doenças são geralmente causadas por mutações em único

gene que resulta no surgimento da doença, apresentando uma herdabilidade que pode ser facilmente traçada durante as gerações (Chakravarti e Little, 2003). A diversidade de mutações em cada locus é alta, cada mutação é rara, pode-se afirmar que estas ocorreram recentemente na história humana e cada mutação é necessária e suficiente para causar o fenótipo de interesse (Chakravarti, 1999). Desordens que seguem esse padrão são raras. No entanto, uma grande parte das doenças que tem um componente genético seguem um padrão de herança poligênica. Nestas, muitos genes envolvidos com o aparecimento do fenótipo, não sendo possível definir um gene principal. Mutações nesses genes são comuns e apresentam apenas um pequeno efeito (Chakravarti e Little, 2003). Esses genes podem agir de forma aditiva, aumentando a susceptibilidade à doença. Este modelo requer também a existência de um limiar de susceptibilidade, a partir do qual a doença passa a ocorrer. Em indivíduos acometidos, esse limiar pode ser atingido através de diferentes combinações de fatores de risco genéticos e ambientais. Dessa forma, a presença isolada de um alelo que predisponha à doença pode não ser nem necessária ou mesmo suficiente para que esta ocorra (Conneally, 2003).

Os estudos científicos de genética em esquizofrenia iniciaram em 1916 (Kendler e Zerbin-Rudin, 1996; Kendler e cols, 1996) embora na oitava edição de seu livro texto, Kraeplin (1913) já descrevera que cerca de 70% de seus pacientes com *dementia pracox* na *Heilberg Clinic* (1891 – 1899) apresentavam história familiar de psicose (Shorter, 1997). Na análise apresentada por Zerbin-Rudin (1967), o risco para filhos de pacientes com esquizofrenia desenvolverem a doença era próxima de 15 vezes maior (12,3%) que a população em geral; irmãos e parentes cerca de 10 vezes (8,5% e 8,2%, respectivamente); para tios (2%); sobrinhos (2,2%) e netos (2,8%) (Tsuang e Vandermey, 1980). Em onze estudos em gêmeos conduzidos entre 1928 e 1972 (Hamilton, 1976), as taxas de concordância em monozigotos variaram de 35% a 69% e em dizigóticos de 0% a 26%. Dados de McGue e Gottesman (1989) mostraram uma maior concordância entre gêmeos, atingindo 80% para monozigóticos e 50% entre os dizigóticos.

Atualmente, a existência de um componente genético na etiologia da esquizofrenia é clara, sendo que estudos mostram repetidamente a presença de um maior risco de incidência entre parentes de esquizofrênicos e que isto deve-se, em sua maior parte, a um componente genético (Gottesman e Shields, 1967). Análises complexas de segregação rejeitaram os modelos monogênicos, sustentando a hipótese de herança poligênica. Dados provenientes de estudos epidemiológicos em genética também relatam que, assim como em outras doenças, esquizofrenia

tem um padrão de transmissão complexo. Entretanto, o número de *loci* de susceptibilidade, o risco da manisfestação da doença para cada locus, a grande heterogeneidade genética e o grau de interação entre esses *loci* são ainda desconhecidos.

O componente genético em esquizofrenia já foi alvo de diversas revisões de literatura (Owen e cols, 2004; Owen e cols, 2005; Riley e Kendler, 2006; Craddock e cols, 2006). Desde o primeiro estudo de ligação realizado por Sherrington e cols (1988), diversos outros estudos descreveram regiões cromossômicas que poderiam abrigar genes associados com a esquizofrenia, sendo que mais de dez diferentes locos cromossômicos já foram relacionados à esquizofrenia (Mirnics e cols, 2001). Alguns dos mais relevantes, comprovados por um maior número de estudos, estão localizados nos braços cromossômicos 1q, 2p, 5q, 6p, 8p, 10p, 17p, 20q e 22q (Brzustowicz e cols, 2000; Freedman e cols, 2001; Gurling e cols, 2001; De Lisi e cols, 2002; Mimmack e cols, 2002; Straub e cols, 2002; Lerer e cols, 2003; Lewis e cols, 2003; Ekelund e cols, 2004; Sklar e cols, 2004; Hamshere e cols, 2006; Suarez e cols, 2006). Os estudos utilizando a estratégia de genes candidatos apresentam entre os genes mais analisados e com resultados positivos replicados destacam-se disbindina 1 - DTNBP1 (6p22.3) (Straub e cols, 2002), neuregulina 1 – NRG1 (8p12) (Stefansson e cols, 2002), ativador da D-aminoácido oxidase – DAOA/G72 (13q33.2-q34) (Chumakov e cols, 2002), regulador tipo 4 da proteína G sinalizadora - RGS-4 (1q23.3) (Chowdari e cols, 2002), catecol-orto-metiltransferase – COMT (22q11.21) (Glatt e cols, 2003); prolina desidrogenase - PRODH (22q11.21) (Jacquet e cols, 2002) e *disrupted in schizophrenia 1* - DISC1 (1q42.1) (Millar e cols, 2000).

1.4 – Tratamento da esquizofrenia

A esquizofrenia é geralmente tratada com uma combinação de psicoterapia e ajustes sociais, bem como administração de fármacos. As propostas terapêuticas para a esquizofrenia mudou drasticamente nos últimos 100 anos. Inicialmente, formulações como cocaína (Becker, 1921), manganês (Reed, 1929), óleo de castor (Ingham, 1930) e injeções de óleo sulfúrico para indução de febres (Croce, 1932; Lehmann, 1993) foram utilizadas. Outros tratamentos incluíam terapia do sono e coma induzido por insulina (Ban, 2001). O primeiro tratamento bem aceito e amplamente utilizado para esquizofrenia foi a clorpromazina (Ban, 2002). A clorpormazina fora sintetizada em 1950 (Charpentier e cols, 1952) e introduzida para o uso clínico em 1952 (Delay e cols, 1952) Na mesma época, enquanto iniciava-se a utilização da clorpromazina na Europa, a reserpina fora sintetizada (1952) e introduzida na prática clínica (1954) na América do Norte (Muller e cols, 1952; Delay e cols, 1954). Porém, à reserpina restaria apenas o interesse histórico e a utilidade como ferramenta farmacológica, sendo a clorpromazina considerada o primeiro dos antipsicóticos, sendo esta uma fenotiazina. Em 1958, uma nova classe foi sintetizada, a das butirofenonas, tendo como protótipo o haloperidol (Janssen, 1996), sendo introduzida na clínica em 1959 (Divry e cols, 1959). Esses fármacos foram inicialmente denominados neurolépticos, curiosamente não devido aos seus efeitos terapêuticos, mas sim devido aos seus efeitos colaterais, os efeitos extrapiramidais. Dentro desse grupo de antipsicóticos conhecidos como típicos ou de primeira geração os mais utilizados atualmente são: clorpromazina, promazina, haloperidol, tioridazina, estelazina, trifluroperazina, tiotixene e sulpirida (Kapur e Remington, 2001).

As duas últimas décadas testemunharam mudanças significativas na utilização de terapia com antipsicóticos em todo mundo. A introdução sucessiva de onze antipsicóticos atípicos ou de segunda geração (clozapina, amilsulprida, zotepina, risperidona, olazapina, quetiapina, sertindol, ziprasidona, aripiprazol, perospirona e paliperidona) criou um otimismo entre clínicos e pacientes sobre o que poderia ser alcançado em relação à eficácia terapêutica desse grupo de drogas. Assim como os 51 antipsicóticos típicos ou de primeira geração (entre eles a clorpromazina) que estão comercialmente disponíveis no mundo, estes 11 princípios ativos são, ao menos, tão eficazes na redução de sintomas típicos como ilusões, alucinações e pensamento desorganizado (Tandon e cols, 2008). Os atípicos são considerados por alguns clínicos mais

interessantes que os típicos por apresentarem maior espectro de atuação (particularmente em relação aos sintomas negativos, cognitivos e relacionados ao humor) e maior segurança em relação à manifestação de efeitos colaterais motores agudos e de longa duração (Moller, 2000; Kapur e Remington, 2001; Meltzer, 2004; Tandon, 2007). Em consequência disso, há um consenso entre médicos e associações médicas recomendando o uso desses novos agentes (Kane e cols, 2003; Miller e cols, 2004; Lehman, 2004; Falkai e cols, 2005). Embora criado tamanho entusiasmo com a introdução dos atípicos, os governos se tornaram receiosos com o aumento significativo dos custos em torno dessa classe de medicação. As despesas globais com medicações antipsicóticas multiplicaram mais de 20 vezes na última década (de 0,5 bilhão para 15 bilhões por ano). Este aumento foi causado em sua maior parte pelo fato de que os atípicos são de cinco a 30 vezes mais caros que as drogas típicas (Hoenberg e Goetz, 2006).

A eficácia terapêutica da clozapina já fora comparada em relação a outras drogas atípicas. No recente estudo no Reino Unido denominado CUtLASS (do inglês "*Cost Utility of the Latest Antipsychotics in Severe Schizophrenia"*) 136 pacientes exibindo uma reposta insatisfatória a dois ou mais agentes antipsicóticos receberam aleatoriamente clozapina ou outro atípico e a qualidade de vida fora acompanhada por um ano (Lewis e cols, 2006). Os resultados mostraram que a clozapina foi mais eficaz que os outros atípicos avaliados de forma significativa com referência à redução de sintomas (p=0,01). Fora igualmente observado de forma quase estatisticamente significativa (p=0.08) uma maior melhora na qualidade de vida neste grupo de pacientes. Esse estudo corrobora com outros que igualmente suportam uma superioridade terapêutica da clozapina em relação a outros princípios atípicos (Kane e cols, 1988; Chakos e cols, 2001; Tuunainen e cols, 2002). Os resultados de outro estudo clínico recente, o americano CATIE (do inglês "*Clinical Antipsychotic Trial of Intervention Effectiveness*") também apresentam uma melhor resposta á clozapina em pacientes refratários (McEvoy e cols, 2006). Em contraste com os dados que suportam uma superioridade clínica da clozapina em relação aos típicos e outros atípicos em pacientes refratários e naqueles com alta taxa de tentativa de suicídio (Meltzer e cols, 2003), não há evidências de uma maior eficácia da clozapina no tratamento do primeiro episódio psicótico (Lieberman e cols, 2003) ou mesmo em outras populações de pacientes.

As diferenças clínicas observadas entre típicos e atípicos podem, ao menos em parte, serem atríbuidas aos mecanismos de ação dessas drogas. Enquanto os típicos apresentam alta

afinidade de ligação *in vivo* com receptores de dopamina do tipo 2 (DRD2) e esse potencial de ligação apresenta relação com a eficácia clínica de cada droga desse grupo; a clozapina, o protótipo dos atípicos, apresenta como alvos diversos receptores, não se restringindo somente ao sistema dopaminérgico. Evidências apontam que ao menos os sistemas serotonérgico, histaminérgico, adrenérgico e colinérgico seriam modulados pelos atípicos. Há a hipótese de que o bloqueio dos receptores de serotonina 2A (5-HT_{2A}) e o bloqueio preferencial de subtipos específicos de receptores da dopamina se constituem como um importante mecanismo para a eficácia dos antipsicóticos atípicos no tratamento dos sintomas negativos (Möller, 2003). Esses dois grupos também diferem entre si em relação à incidência dos efeitos colaterais. Enquanto pacientes em uso de típicos tendem a apresentar efeitos extrapiramidais, tal como a discinesia tardia (DT); os usuários dos atípicos são mais frequentemente acometidos com desbalanços metabólicos, sendo notáveis ganho de peso e uma maior incidência de diabetes nesse grupo. Assim como na resposta a esses medicamentos, a DT e o ganho de peso induzido por antipsicóticos parecem apresentar um componente genético que influencia a incidência bem como a gravidade desses efeitos colaterais.

A história da farmacogenética inicia-se na década 1950 após Arno Motulsky enunciar que: "traços herdados poderiam explicar as diferenças tanto no efeito das drogas quanto na presença de efeitos colaterais", e evoluiu paralelamente com a história da genética. Diversos resultados mostram que a resposta a antipsicóticos pode ter um componente genético (Arranz e De Leon, 2007; Malhotra e cols, 2007). Em 2005, o órgão regulador americano *Food e Drug Administration (FDA)* aprovou para uso clínico um *chip* com o nome commercial de AmpliChip® CYP450 fabricado pela Roche. Este chip possibilita o teste de dois genes polimórficos, o do citocromo P450 2D6 (CYP2D6) e do citocromo P450 2C19 (CYP2C19), enzimas que são responsáveis pela metabolização de várias drogas antidepressivas e antipsicóticas (De Leon e cols, 2006).

Estudos farmacogenéticos têm analisado primariamente os medicamentos atípicos, em especial a clozapina, talvez porque seja mais fácil o acesso ao sangue desses pacientes, uma vez que estes precisam ser monitorados quanto à agranulocitose ou por causa da superior eficácia clínica em populações de paciente resistentes ao tratamento. Análises farmacogenéticas da resposta à clozapina têm utilizado a estratégia de estudos de associação com genes candidatos, usando como genes candidatos os receptores dopaminérgicos e serotonérgico (Malhotra e cols,

2004). Estes seriam candidatos com forte racionalidade biológica já que clozapina apresenta alta afinidade com estes receptores.

Arranz e colaboradores, em 1995, chamaram a atenção para o receptor 5-HT_{2A} reportando uma associação significativa com o alelo 102C e uma pior resposta à clozapina numa população de 149 pacientes com esquizofrenia. Entretanto, esse resultado não foi consistentemente replicado por uma série de outros estudos, sendo que alguns incluíram outros antipsicóticos (Masellis e cols, 1995; Burnet e Harrison, 1995). A variação T102C no 5-HT_{2A} pode ser considerada como um fraco candidato uma vez aqui a troca das bases não implica na troca de aminoácidos na proteína e nenhuma função fora até então descrita para essa mudança de bases (Masellis e cols, 1995). A variação His452Tir, que não foi encontrada em desequilíbrio de ligação com o T102C (Malhotra e cols, 1996), aparenta produzir alterações funcionais *in vitro*. Entretanto, não fora relatada nenhuma forte associação dessa variante com a resposta à antipsicóticos (Nothen e cols, 1995). A variante -1438G/A já foi analisada nesse mesmo contexto e não foram relatadas associações (Arranz e cols, 1998a).

Outros genes no sistema serotonérgico foram avaliados, entre eles os receptores 5-HT_{1A} (Masellis e cols, 2001), 5-HT_{2C} (Sodhi e cols, 1995), 5-$HT_{3A/B}$ (Arranz e cols, 2000b), 5-HT_{5A} (Birkett e cols, 2000), 5-HT_6 (Yu e col, 1999), 5-HT_7 (Masellis e cols, 2001), o transportador de serotonina - 5HTT (Arranz e cols, 2000a; Tsai e cols, 2000) e triptofano hidroxilase – TPH (Anttila e cols, 2007). Embora alguns resultados apresentem associações positivas, há somente uma fraca indicação de que esses genes são associados com a resposta à antipsicóticos. Genes no sistema dopaminérgico também foram extensivamente analisados, tais como receptores DRD1 (Potkin e cols, 2003), DRD2 (Arranz e cols, 1998b), DRD3 (Shaikh e cols, 1996), DRD4 (Shaikh e cols, 1993) e o transportador de dopamina – DAT (Szekeres e cols, 2004).

Diversos outros genes já foram igualmente reportados em análises de associação com a resposta aos antipsicóticos, entre eles a subunidade 2B do receptor NMDA de glutamato - GRIN2B (Hong e cols, 2001a), o receptor de histamina do tipo 1 e 2 – H_1 e H_2 (Mancama e cols, 2002), receptores α1 e α2 adrenérgicos (Bolonna e cols, 2000; Tsai e cols, 2001; De Luca e cols, 2005), transportador de norepinefrina – NET (Meary e cols, 2007); receptor de neurotensina (Huezo-Diaz e cols, 2004), fator neurotrófico derivado do cérebro – BDNF (Hong e cols, 2003), antígeno HLA-A1 (Lahdelma e cols, 2001), fator de necrose tumoral alfa (Tsai e cols, 2003), apolipoproteína E (Hong e cols, 2000), COMT (Yamanouchi e cols, 2003), subunidade β3 da

proteína G – GNB-3(Muller e cols, 2005a), RGS-2 (Greenbaum e cols, 2007), RGS-4 (Kampman e cols, 2006), proteínas de ligação a fator solúvel sensível a N-etilmaleimida SNAP-25 (Muller e cols, 2005), resistência múltipla à drogas MDR-1/ABCB1 (Yasui-Furukori e cols, 2006), NEF3(Strous e cols, 2007), proteína quinase B – PKB/Akt1 (Xu e cols, 2007), glicogênio sintase quinase 3 isoforma β (Souza e cols, 2008), colina acetiltransferase – ChAT (Mancama e cols, 2007), NOTCH4 (Anttila e cols, 2004); NRG-1 (Kampman e cols, 2004), enzima conversora de angiotensina – ECA (Illi e cols, 2003) e glicoproteína P – PGP (Lin e cols, 2006).

1.4.1 – Discinesia tardia induzida por antipsicóticos

A DT é uma síndrome extrapiramidal induzida por antipsicóticos, caracterizada por movimentos involuntários, anormais e repetitivos localizados principalmente na região orofacial, tronco, extremidades inferiores e superiores, podendo acometer inclusive o sistema respiratório. O termo DT foi introduzido por Faurbye (1964), como tardia enfatizando a cinética temporal até a apresentação de movimentos involuntários, já que esse efeito colateral é causado por uma longa utilização de antipsicóticos. O diagnótico apresentado pelo DSM-IV (American Psychiatric Association, 2000) requer pelo menos três meses de exposição a essas drogas. A DT é potencialmente irreversível com a descontinuação do uso da medicação.

Os dados de prevalência são difícies de interpretar uma vez que apresentam estudos em populações heterogêneas e formas diagnóticas diferentes. Em uma revisão 56 estudos entre 1959 e 1979, Kane e Smith (1982) encotraram uma prevalência de 0,5 até 65%. Em 1992, Yassa e Jeste reportaram uma prevalência de 24% em 39187 pacientes de 76 estudos. Dois outros estudos utilizando mesmo critérios diagnósticos, Woerner e cols (1991) e Muscettola e cols (1993), apresentaram prevalência de 23,4% e 19,1%, respectivamente. Dados mais recentes mostram que DT acomete pelo menos 20% dos indivíduos em uso de antipsicóticos, com taxas de incidência para novos casos de aproximadamente 3 a 5% ao ano (Kane, 2001). Essa incidência parece ocorrer de maneira cumulativa e chegar a 30% entre os idosos expostos ao uso crônico de antipsicóticos. Além dos antipsicóticos, outros fatores que têm sido relacionados ao aparecimento e prognóstico da discinesia tardia são: idade, gênero feminino, co-morbidade psiquiátrica, presença de outros transtornos extrapiramidais na fase aguda do tratamento com antipsicóticos e diabetes (Kane, 2001).

Em função da complexidade dessa síndrome, diversas teorias fisiopatológicas têm sido sugeridas, de maneira geral implicando um ou mais neurotransmissores no aparecimento dos sintomas de DT. No passado, acreditava-se que o uso crônico de antipsicóticos provocasse uma hipersensibilidade dopaminérgica na região nigro-estriatal que levaria ao surgimento dos sintomas de DT. Entretanto, essa hipersensibilidade não explica a susceptibilidade individual para desenvolver discinesia tardia e passou-se então a investigar a ocorrência de possíveis alterações concomitantes nos neurotransmissores colinérgicos, GABAérgicos e serotoninérgicos. Além disso, tem-se sugerido que o uso crônico desses medicamentos causaria uma

superprodução de radicais livres de oxigênio com conseqüente degeneração neuronal (Cadet e Lohr, 1989). Essa degeneração, a princípio reversível, resultaria em lesões irreversíveis dos neurônios nigroestriatais, com conseqüente morte celular, e o aparecimento da DT.

Há uma forte concordância em relção a incidência de DT entre parentes de primeiro-grau em uso de antipsicóticos (Youssef e cols, 1989; Müller e cols, 2001). Em função de que as primeiras explicações para o surgimento de DT estarem vinculadas a uma maior atividade do sistema dopaminérgico, em especial no gânglio basal, e as drogas típicas (mais frequentemente associadas a esse efeito colateral) serem antagonista de receptores dentro desse sistema, genes que codificam moléculas envolvidas com a propagação do sinal dopaminérgico foram primeiramente analisadas em estudos de associação (Ozdemir e cols, 2001). Confirmando a plausibilidade biológica, o DRD2 apresentou resultados positivos, especialmente com a variante C939T (Chen e cols, 1997). Da mesma forma, sugere-se associação significante com outros genes do sistema dopamineérgico: DRD1 (Srivastava e cols, 2006); DRD3 (Rietschel e cols, 1993). DRD4 (Rietschel e cols, 1996) e o DAT (Segman e cols, 2003) já foram igualmente analisados entretanto não apresentaram resultados significativos.

Genes em outros sistemas de neurotransmissão, tais como serotonérgico, e genes envolvidos com o processamento do estresse oxidativo (Thelma e cols, 2007) foram também reportados. Entre eles encontra-se resultados com: 5-HT_{2A} (Segman e cols, 2001); 5-HT_{2C} (Segman e cols, 2000), 5-HT_6 (Ohmori e cols, 2002), 5-HTT (Chong e cols, 2000), TPH (Segman e cols, 2003), COMT (Herken e cols, 2003); monoamino oxidase – MAO (Matsumoto e cols, 2004), GRIN2B (Liou e cols, 2007), receptor de adenosina 2A – A_{2A} (Hong e cols, 2005), receptor opióide μ e Δ (Ohmori e cols, 2001), receptor de estrógeno (Lai e cols, 2002), GNB-3 (Lee e cols, 2007), PGP (de Leon e cols, 2005), BDNF (Liou e cols, 2004), mangânes superóxido dismutase – MnSOD (Hori e cols, 2000), NAD(P)H quinona oxiredutase - NQO1 (Pae e cols, 2004); glutationa peroxidase – GPX1 (Shinkai e cols, 2006); óxido nítrico sintase – NOS (Shinkai e cols, 2004), fenilalanina hidroxilase (Richardson e cols, 2006); glutationa-S-transferases – GSTM1 e GSTT1 (Pae e cols, 2004), ECA (Segman e cols, 2002), CYP1A2 (Basile e cols, 2000); CYP2D6 (Arthur e cols, 1995), CYP3A4 (Tiwari e cols, 2005) e CYP3A5 (de Leon e cols, 2005).

1.4.2 – Ganho de peso induzido por antipsicóticos

O ganho de peso é um sério problema em pacientes usando antipsicóticos (Malhotra e cols, 2004; Müller e cols, 2006). Evidências mostram que os antipsicóticos interagem no sistema neuroendócrino, levando a efeitos colaterais como aumento do apetite, obesidade, hiperglicemia e diabetes (Bernstein, 1987; Henderson e cols, 2000). O excesso de peso é um evento comum nesses pacientes, tendo sido demonstrado que os mesmos apresentam um índice de massa corporal significativamente maior do que os pacientes psiquiátricos sem o diagnóstico de esquizofrenia e do que a população geral (Allison e Casey, 2001). A magnitude do ganho de peso varia conforme os medicamentos e a dosagem, sendo que alguns se destacam por ganhos de peso de 1,5 a 8,8 kg em períodos de 6 meses (Allison e cols, 1999; Goudie e cols, 2005). Vários estudos convergem e sugerem que alguns antipsicóticos atípicos implicam em ganho de peso significativamente maior após a administração em curto e em longo prazo, quando comparados com antipsicóticos típicos (Henderson, 2007).

O excesso de peso corporal aumenta intensamente o risco de mortalidade e morbidade de vários transtornos clínicos, incluindo hipertensão, dislipidemia, diabetes melito tipo II, doenças cardíacas, doenças da vesícula biliar, osteoartrite, apnéia do sono, problemas respiratórios e cânceres de endométrio, mama, próstata e cólon, reduzindo ainda mais a sobrevida e a qualidade de vida dos portadores de esquizofrenia (NIH, 1998). Desta forma, há uma atenção para se adequar a prescrição ao perfil do paciente, questionando sobre outros fatores de risco, como hipertensão, diabetes prévia, idade maior que 50 anos, raça e história familiar. Além disso, o monitoramento dos níveis glicêmicos e ponderais deve ser feito para orientar estratégias no tratamento destas alterações (Meltzer, 2001). Apesar de recente e pouco explorado, Wehmeier e colaboradores e Theisen e colaboradores, ambos em 2005, apresentaram dados que mostram concordância de ganho de peso induzida por antipsicótico em gêmeos monozigóticos e/ou pares de irmãos do mesmo sexo.

A maioria dos estudos genéticos que avaliaram a indução de ganho de peso por antipsicóticos examinaram genes que codificam proteínas do sistema nervoso central, especialmente receptores de neurotransmissores. A seleção de genes candidatos a serem analisados nesse contexto baseia-se primariamente nas conhecidas bases neurobiológicas da saciedade. Por outro lado, é possível pensar que a medicação possa também operar em

mecanismos periféricos, tais como o controle do metabolismo e tônus muscular modulando assim a queima de calorias e/ou diretamente alterando a lipogênese (Basile e cols, 2001). Grande parte dos estudos tem se focado no sistema serotonérgico que é conhecido como controlador da saciedade. Os sinais de controle de saciedade convergem no hipotálamo provenientes de diversas áreas do corpo, incluindo receptores gustativos, olfatórios, gástricos, intestinais e hepáticos. Outros pontos que fortalecem a idéia de um papel da serotonina nesse contexto surgem em torno da capacidade dessa amina modular o comportamento alimentar em diversos modelos analisados. De uma forma geral, o aumento da concentração de serotonina estaria relacionado a uma diminuição do comportamento alimentar, sendo que a relação inversa é igualmente verdadeira (Davis e Faulds, 1996). Alguns receptores e o transportador de serotonina já foram analisados nesse contexto: 5-HT_{1A} (Basile e cols, 2001), 5-HT_{2A} (Hong e cols, 2001b), 5-HT_{2C} (Rietschel e cols, 1997), 5-HT_6 (Hong e cols, 2001b), 5-HTT (Hong e cols, 2001b). Estudos exploratórios com: DRD1 (Lane e cols, 2006), DRD2 (Lane e cols, 2006), DRD3 (Lane e cols, 2006), DRD4 (Rietschel e cols, 1996), H_1 (Basile e cols, 2001), H_2 (Basile e cols, 2001), SNAP-25 (Müller e cols, 2005b), CYP2D6 (Ellingrod e cols, 2002), CYP1A2 (Basile e cols, 2001), receptores adrenérgicos (Basile e cols, 2001), leptina e seu receptor (Zhang e cols, 2003), neuropeptídeo Y e seus receptores (Ruaño e cols, 2007), paraoxonase 1 (Ruaño e cols, 2007), apoliproteínas A4 e E (Ruaño e cols, 2007), PGP (Lin e cols, 2006), TNF-α (Zai e cols, 2006), BDNF (Lane e cols, 2006) e GNB-3 (Tsai e cols, 2004) foram reportados.

2. Objetivos

- Determinar se há participação genética dos receptores α do fator neurotrófico derivado de glia (GDNF) na esquizofrenia e na resposta ao tratamento de clozapina.

- Estudar a prediposição genética à esquizofrenia e à resposta a clozapina associada a marcadores no gene da GSK-3.

- Avaliar o papel do gene NALCN na genética de esquizofrenia, resposta ao tratamento, discinesia tardia induzida por antipsicóticos e ganho de peso induzido por clozapina.

- Analisar se a variante C825T no gene da GNB-3 está associado com o ganho de peso induzido por antipsicóticos e no índice de massa corpórea

3. Artigos

3.1. Association study of GSK3 gene poymorphisms with schizophrenia and clozapine response

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ORIGINAL INVESTIGATION

Association study of GSK3 gene polymorphisms with schizophrenia and clozapine response

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Abstract
Rationale A number of human and animal studies implicate GSK3 in the pathophysiology and genetics of schizophrenia. In general, the data suggest that phosphorylation levels of GSK3β are reduced in schizophrenia, resulting in increased GSK3β activity. Since GSK3β regulation is altered in schizophrenia, polymorphic variation in this gene may affect susceptibility to schizophrenia or treatment response.
Objective To analyze *GSK3β* genetic variants for association with schizophrenia and clozapine response.
Materials and methods We examined *GSK3β* markers in 185 matched case–control subjects, 85 small nuclear families, and 150 schizophrenia patients treated with clozapine for 6 months.
Results Three markers (rs7624540, rs4072520, and rs6779828) showed genotypic association with schizophrenia in the case–control sample. We did not observe any family and clozapine response association with a specific allele, genotype, or haplotype.
Conclusions Our results suggest that GSK3β polymorphisms might be involved in schizophrenia risk but do not appear to play a significant role in clozapine response.

Keywords Schizophrenia · Clozapine response · GSK3β · Wnt signaling · Genetic association · Family-based association test

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Introduction

Schizophrenia is a devastating psychiatric disorder that affects 1% of the population, and pharmacological treatment is only partially effective for the majority of patients. There are currently no clear criteria by which to predict treatment response or to select the optimal drug that will have the best efficacy and minimal side effects (Seeman 2002; Ananth et al. 2004). Approximately 10–20% of patients do not respond to antipsychotic drug treatment, and an additional 20–30% do respond early on eventually relapse; others develop serious side effects that cause them to discontinue medication altogether (Malhotra et al. 1993, 2004; Basile et al. 2002). Pharmacogenetic studies may help to identify genetic variants that modulate treatment response or side-effect profiles, with the eventual objective of applying this knowledge to clinical practice.

Glycogen synthase kinase 3 (GSK3) phosphorylates, and thereby regulates, a large number of substrates, with over

50 known to date (Jope et al. 2007). Four GSK3 regulatory mechanisms have been described: phosphorylation, protein complexes, localization, and substrate phosphorylation; and in combination, these can provide substrate-specific regulation of GSK3 activity. The most well-defined regulatory mechanism is inhibition of GSK3 activity by phosphorylation of serine-9 in the isoform GSK3β or serine-21 in GSK3α (Woodgett 1990; Sutherland et al. 1993). The phosphatidylinositol 3-kinase/Akt signaling pathway is activated by insulin and many growth factors, and this pathway can also regulate GSK3 through Akt phosphorylation of GSK3 on these same serine residues. Protein kinase C and protein kinase A are also able to phosphorylate these regulatory serines (Shin et al. 2002; Jope and Johnson 2004).

There is support for the hypothesis that alterations in GSK3 are connected with schizophrenia and its treatment, mainly in the areas of altered dopaminergic activity and disrupted neurodevelopment. All antipsychotics bind to dopamine D2 receptors with an affinity inversely proportional to the clinically effective dose (Seeman and Lee 1975). Some antipsychotics with very high D2 receptor affinity such as haloperidol bind with low affinity to other related G-protein-coupled neurotransmitter receptors (Kapur and Seeman 2001). In contrast, some antipsychotics such as clozapine have relatively low D2 receptor affinity but bind strongly to 5-HT2 receptors (Meltzer 1989). Therefore, typical and atypical antipsychotics may regulate GSK3 activity via different signaling pathways. Previously, Kang et al. (2004) demonstrated that although both Dvl and Akt are activated by clozapine, only Dvl is responsible for Ser9-phosphorylation of GSK3 in SH-SY5Y cells. More recently, Roh et al. (2007) compared the phosphorylation at Ser21/9 residues of GSK3 in vivo after acute treatment with haloperidol or clozapine. Acute treatments with both drugs increased pSer21/9-GSK3 in vivo, but they affected upstream regulators differently. Furthermore, Li et al. (2007) recently have reported that clozapine increases pSer9-GSK3β.

Low immunoreactivity and activity of GSK3β was demonstrated post mortem in the frontal cortex of schizophrenia patients (Kozlovsky et al. 2000, 2001, 2004; Beasley et al. 2001; Nadri et al. 2003), but there is at least one negative study on a different brain collection (Nadri et al. 2004). Previous genetic studies have not found significant association between *GSK3β* and schizophrenia in case–control samples (Scassellati et al. 2004; Ikeda et al. 2005; Lee et al. 2006; Szczepankiewicz et al. 2006). However, the potential effect of genetic variation in *GSK3β* on clozapine response has not yet been explored. Thus, our objectives were to examine genetic association between *GSK3β* and schizophrenia (using case–control and nuclear family samples), as well as clozapine response.

Materials and methods

Clinical sample

All recruitment and clinical assessments were conducted with written informed consent and with the explicit approval of our institutional ethics review board. Clinical data and DNA samples were obtained from the probands of 85 small nuclear families, as well as 180 Caucasian patients with a DSM-III-R or DSM-IV diagnosis of schizophrenia. The healthy controls (N=185) were matched for age (±5 years) and sex, 146 male and 73 female cases and the same number of controls, mean age 36±8). The Structured Clinical Interview for DSM-IV Axis I Disorders was administered by trained research assistants to each patient, and diagnosis was supplemented by a review of medical records. The diagnosis was established via consensus procedures incorporating two of the investigators. The controls were screened for current or past history of major psychiatric disorders.

For the clozapine response sample, clinical data from 140 patients with a DSM-III-R diagnosis of schizophrenia (114 Caucasians), almost all of whom were treatment refractory or intolerant of typical antipsychotic therapy (Kane et al. 1988), were obtained at two research clinics: Case Western Reserve University in Cleveland, OH, USA (n=90; 63 males and 27 females, mean age 36±8) and Hillside Hospital in Glen Oaks, NY, USA (n=50; 33 males and 17 females, mean age 35±8). After informed consent was obtained, patients underwent a washout period of 2 to 4 weeks during which, unless clinically necessary, they received no medications before starting clozapine. Clozapine treatment was continued for a minimum of 6 months during which patients were evaluated prospectively. Clozapine blood levels were monitored during the course of treatment to ascertain compliance. Treatment response was evaluated as the percentage score change on the 18-item Brief Psychiatric Rating Scale (BPRS). Treatment response was expressed as a dichotomous variable at 6 months using criteria based on those of Kane (Kane et al. 1988): a reduction of ≥20% on the overall score of the BPRS from the baseline score at enrolment. There were no differences observed between the sites in terms of gender ratio, mean age, mean age of onset, or response ratio. Caucasians and African-American subjects were not significantly different in terms of gender ratio, mean age, mean age of onset, or response ratio (data not shown; Hwang et al. 2005).

Genetic analyses

Genomic DNA was extracted using the high salt method of Lahiri and Nurnberger (1991). We analyzed 12 markers across *GSK3β* (rs6805251, rs4688043, rs7624540,

rs13319151, rs6438552, rs4072520, rs9878473, rs4491944, rs6772172, rs11919783, rs11923196, rs6779828, rs9846422, rs3755557) and two markers after the 3′-untranslated region of the gene (rs9846422, rs3755557). Genotyping was performed by GoldenGate assay (Illumina, San Diego, CA, USA) at The Centre for Applied Genomics at the Hospital for Sick Children in Toronto, ON, Canada. Rs4072520 was re-genotyped in the whole sample using TaqMan® SNP Genotyping Assays from Applied Biosystems as a quality check procedure.

Statistical analyses

Individual markers analyses of responder (control)/non-responder (case) data were performed using χ^2 tests. The statistical program used was the Statistical Package for the Social Sciences, version 10.0.7. We applied the family-based association test (FBAT, version 1.0, Laird et al. 2000) under the assumption of an additive model and PEDSTATS (Wigginton and Abecasis 2005) for Hardy–Weinberg equilibrium in the family data. Linkage disequilibrium (LD) was assessed using Haploview, version 4.0 (Barrett et al. 2005). Haplotype analyses were performed using UNPHASED 3.0.10 (Dudbridge 2003) and Haploview, version 4.0.

Results

Linkage disequilibrium analysis

Pairwise LD between the markers is presented for each sample (Fig. 1). In this study, we defined a haplotype block as a region over which less than 5% of pairwise comparisons among informative markers showed strong evidence of historical recombination (upper confidence bound on D' less than 0.9; Gabriel et al. 2002). We found the same block in each of the case–control, family, and response samples composed of 12 of the 14 markers.

Genotype data

Significant deviations from Hardy–Weinberg equilibrium were observed for rs7624540 (p=0.007), rs4075250 (p=0.004), and rs6779828 (p=0.023) in cases from the case–control sample (Fig. 2). Only two markers in cases showed deviation from Hardy–Weinberg equilibrium at a significance threshold of p<0.01. Cases and controls were compared for genotype and allele frequencies across the markers (see Table 1). The following markers were genotypic distribution associated with schizophrenia: rs7624540 (χ^2=8.28, genotype p=0.016), rs4072520 (χ^2=9.94, genotype p=0.007), and rs6779828 (χ^2=6.83, genotype p=0.033).

In the family samples, no significant deviation from Hardy–Weinberg equilibrium was observed among 81 unrelated individuals (data not shown). Allele frequencies are shown in Table 2. Family-based association results showed no association with schizophrenia. In the clozapine response sample, there were no significant deviations from

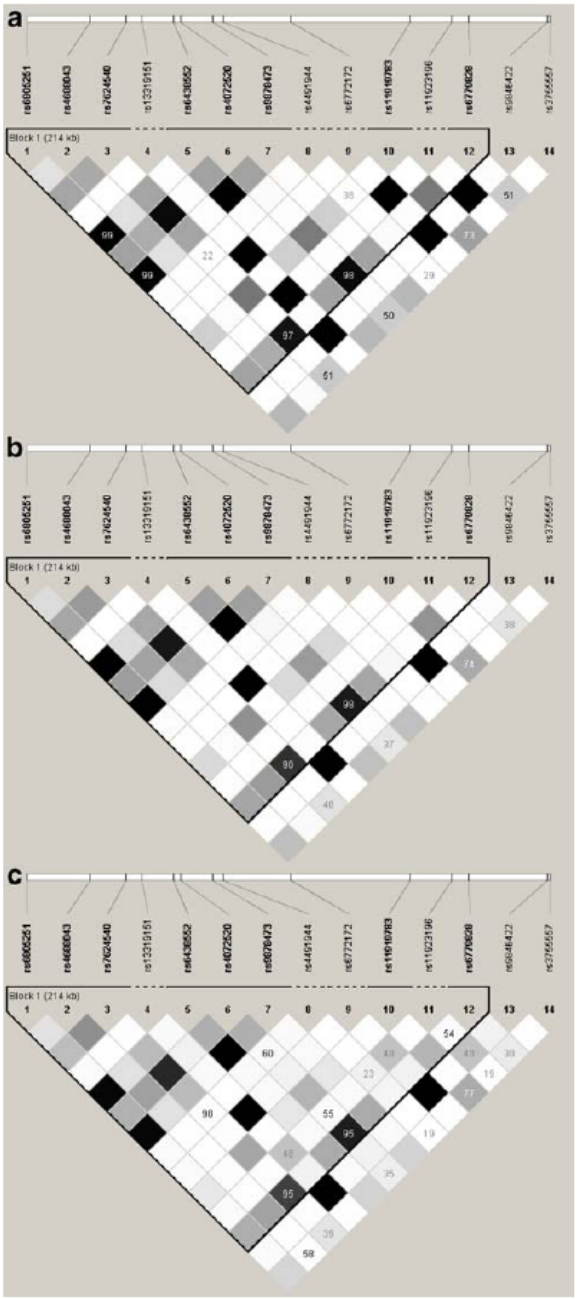

Fig. 1 LD plot for the analyzed markers in case–control, family, and response samples (**a**, **b**, **c**). Values presented are the D'

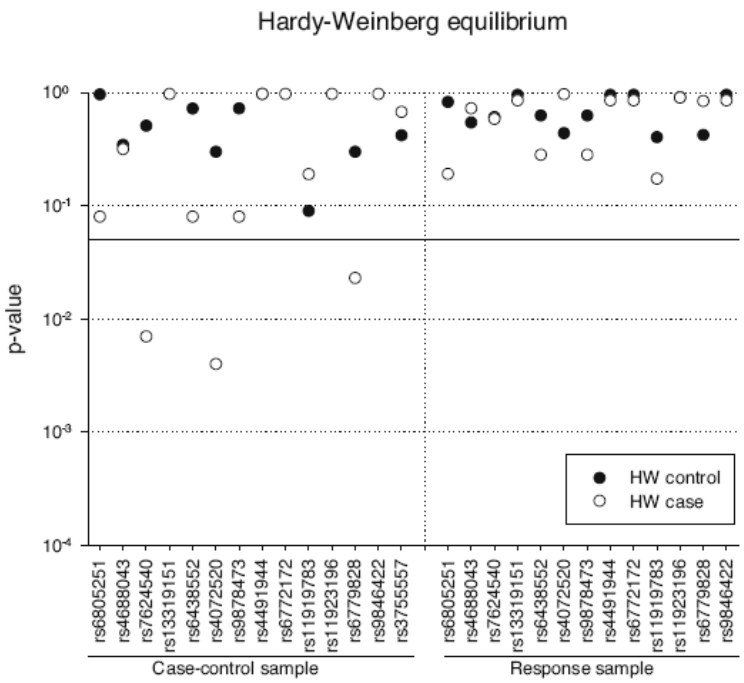

Fig. 2 Hardy–Weinberg equilibrium p values for case–control and responders (cases)-non-responders (controls). The *solid line* represents $p=0.05$

Hardy–Weinberg equilibrium (Fig. 2). Responder/non-responder groups were compared for genotype and allele frequencies across the markers, and no significant differences were observed (see Table 3), including when Caucasian or African-American subjects were analyzed separately (data not shown).

Haplotype analyses

Cases and controls were compared for haplotype frequencies across the *GSK3β* markers. We performed a three-marker window (Durrant et al. 2004), and results did not show association. Allelic occurrence in haplotypes constructed by rs7624540, rs4072520, and rs6779828 (markers that showed single genotypic association) showed an overall trend ($\chi^2=9.22$, $p=0.05$), as well as the A–A–G haplotype ($\chi^2=3.09$ $p=0.078$; after 1,000 permutations $p=0.223$). Furthermore, genotypic occurrence in haplotypes constructed by rs7624540, rs4072520, and rs6779828 showed an overall significant association ($\chi^2=23.79$, $p=0.001$), and the A/A–A/A–A/A haplotype presented the strongest association ($\chi^2=7.98$ $p=0.004$; after 1,000 permutations $p=0.011$). Considering haplotypes in the main LD block, no association was found. In the family sample, no haplotype associations were observed. Haplotype frequencies in clozapine responders and non-responders were compared, and no associations were observed in the whole sample, nor were there associations for specific haplotype blocks when Caucasian or African-American subjects were analyzed separately.

Discussion

This exploratory study examined the association of 14 markers spanning the GSK3β gene and two phenotypes: schizophrenia (case–control and nuclear family samples) and clozapine response. Three individual markers (rs7624540, rs4072520, and rs6779828) and one haplotype (rs7624540, rs4072520, and rs6779828) showed nominally significant association with schizophrenia in our case–control sample. The matched case–control sample has 43.2% power to detect a significant effect with a genotypic relative risk of 2.0 (Purcell et al. 2003). Although gene variants of small effect or those that are rare major determinants of risk for developing schizophrenia would likely not be detected because very large sample size would be required, we have found three positive associations. Additional studies in larger samples are needed to resolve whether *GSK3β* is truly playing a role in schizophrenia, especially after our significant findings. No associations were detected in the nuclear family samples.

Corrections for multiple testing have been a controversial issue (Aickin 1999; Bender and Lange 1999; Perneger 1998), and considering the exploratory nature of this study,

Table 1 Allelic and genotypic frequencies for the case–control association

SNP	Allele frequency						Genotype frequency						Association			
	Control			Case			Control			Case			Allele		Genotype	
		Samples	Percent		Samples	Percent		Samples	Percent		Samples	Percent	Chi square	*p* value	Chi square	*p* value
rs6805251	A	141	38.1	A	138	38.3	AA	27	14.6	AA	21	11.7	0.01	0.950	1.60	0.449
	C	229	61.9	C	222	61.7	AC	87	47.0	AC	96	53.3				
							CC	71	38.4	CC	63	35.0				
rs4688043	A	346	93.5	A	335	93.1	AA	161	87.0	AA	155	86.0	0.06	0.804	0.66	0.797
	G	24	06.5	G	25	06.9	AG	24	13.0	AG	25	14.0				
							GG	00	00.0	GG	00	00.0				
rs7624540	A	70	18.9	A	60	16.7	AA	08	04.3	AA	00	00.0	0.63	0.426	8.28	0.016
	G	300	81.1	G	300	83.3	AG	54	29.2	AG	60	33.3				
							GG	123	66.5	GG	120	66.7				
rs13319151	A	00	00.0	A	01	00.3	AA	00	00.0	AA	00	00.0	1.03	0.867	1.03	0.867
	G	359	100.0	G	359	99.7	AG	00	00.0	AG	01	00.6				
							GG	185	100.0	GG	179	99.4				
rs6438552	A	229	61.9	A	222	61.7	AA	72	38.9	AA	63	35.0	0.01	0.950	2.20	0.333
	G	141	38.1	G	138	38.3	AG	85	45.9	AG	96	53.3				
							GG	28	15.1	GG	21	11.7				
rs4072520	A	71	19.2	A	63	17.5	AA	09	05.0	AA	00	00.0	0.35	0.555	9.94	0.007
	C	299	80.8	C	297	82.5	AC	53	29.0	AC	63	35.0				
							CC	123	65.0	CC	117	65.0				
rs9878473	A	229	61.9	A	222	61.7	AA	72	38.9	AA	63	35.0	0.01	0.950	2.20	0.333
	T	141	38.1	T	138	38.3	AT	85	45.9	AT	96	53.3				
							TT	28	15.1	TT	21	11.7				
rs4491944	A	01	0.03	A	01	0.03	AA	00	00.0	AA	00	00.0	0.01	0.999	0.01	0.984
	T	369	99.7	T	359	99.7	AT	01	00.5	AT	01	00.6				
							TT	184	99.5	TT	179	99.4				
rs6772172	A	359	100.0	A	359	99.7	AA	185	100.0	AA	179	99.4	1.03	0.867	1.03	0.867
	G	00	00.0	G	01	00.3	AG	00	00.0	AG	01	00.6				
							GG	00	00.0	GG	00	00.0				
rs11919783	A	44	11.9	A	32	08.9	AA	05	02.7	AA	00	00.0	1.76	0.184	5.01	0.082
	G	326	88.1	G	328	91.1	AG	34	18.4	AG	32	17.8				
							GG	146	78.9	GG	148	82.2				
rs11923196	A	00	00.0	A	01	00.3	AA	00	00.0	AA	00	00.0	1.03	0.867	1.03	0.867
	T	359	100.0	T	359	99.7	AT	00	00.0	AT	01	06.6				
							TT	185	100.0	TT	179	99.4				
rs6779828	A	71	19.2	A	62	17.2	AA	09	04.9	AA	01	00.6	0.47	0.491	6.83	0.033
	G	299	80.8	G	298	82.8	AG	53	28.6	AG	60	33.3				
							GG	123	66.5	GG	119	66.1				
rs9846422	A	359	100.0	A	359	99.7	AA	185	100.0	AA	179	99.4	1.03	0.867	1.03	0.867
	G	00	00.0	G	01	00.3	AG	00	00.0	AG	01	06.6				
							GG	00	00.0	GG	00	00.0				
rs3755557	A	312	84.3	A	311	86.4	AA	133	71.9	AA	135	75.0	0.62	0.430	0.63	0.728
	C	58	15.7	C	49	13.6	AC	46	24.9	AC	41	22.8				
							CC	06	03.2	CC	04	02.2				

Table 2 Family-based association test results

SNP	Allele	Frequency	Family	*S*	*E*(*S*)	Var(*S*)	*Z*	*p*
rs11919783	A	0.125	13	10	7.333	3.222	1.486	0.137
rs11923196	A	0.003	00	–	–	–	–	–
rs13319151	A	0.005	01	–	–	–	–	–
rs3755557	A	0.819	17	22	23.833	4.694	−0.846	0.397
rs4072520	A	0.238	29	19	17.783	7.764	0.437	0.662
rs4491944	A	0.010	02	–	–	–	–	–
rs4688043	A	0.885	13	20	18.217	3.820	0.912	0.362
rs6438552	A	0.561	34	41	41.950	10.264	−0.297	0.767
rs6772172	A	0.995	01	–	–	–	–	–
rs6779828	A	0.225	27	17	16.117	7.042	0.333	0.739
rs6805251	A	0.436	34	29	28.050	10.264	0.297	0.767
rs7624540	A	0.226	27	18	16.783	7.264	0.451	0.652
rs9846422	A	0.995	01	–	–	–	–	–
rs9878473	A	0.562	34	41	41.950	10.264	−0.297	0.767

S Test statistic for observed number of alleles, *E* expected value of *S* under null hypothesis, *Var(S)* variance between the observed and expected transmission

without prespecified hypotheses for most of our markers, the individually significant associations require independent confirmation (Bender and Lange 1999). No associations remain significant after Bonferroni correction (Bonferroni-corrected threshold $p<0.003$). Bonferroni correction is based on the assumption that tests are independent. This is a very conservative adjustment for these data due to the high levels of LD. Applying the Nyholt (2004) correction (Nyholt-corrected threshold $p<0.006$), a nearly significant result for rs4072520 (genotype $p=0.007$) is found. Nyholt showed that significant results occurring after calculating the adjusted alpha value that are not significant with standard Bonferroni or Sidak correction indicate substantial non-independence (i.e., strong intermarker LD) among the tested markers. Therefore, permutation testing was applied as strong LD can be seen (Fig. 1). After 1,000 permutations, the rs4072520 association remains significant ($p<0.008$).

All markers that showed nominal individual significant association with schizophrenia deviated ($p<0.05$) from Hardy–Weinberg equilibrium among the cases. Although the standard χ^2 test often has been used, the Fischer exact test is preferred when genotype counts are low since it does not rely on the χ^2 null distribution approximation (Guo and Thompson 1992; Wigginton et al. 2005). With the Fisher exact test, significant deviations from Hardy–Weinberg equilibrium were observed in cases (rs7624540 $p=0.005$; rs4075250 $p=0.002$; rs6779828 $p=0.032$). At a significance threshold of $p<0.01$, just two markers in the cases show deviation from the Hardy–Weinberg equilibrium (HWE). Trikalinos et al. (2006) concluded that HWE should be routinely and transparently assessed in gene–disease association studies. An outlier marker with extreme HWE disequilibrium usually results from genotyping error, so it has also been used to detect such errors (Hoh et al. 2001). None of the markers appears to be an outlier according to the HWE statistics, so HWE resulting from genotyping error is not a likely explanation for the observed results. In our sample, it is more likely to be an association finding in a recessive genetic model. The detection of HWE disequilibrium has recently been shown to be useful as a method for detecting gene–phenotype association (Feder et al. 1996; Nielsen et al. 1998; Hoh et al. 2001; Lee 2003; Hao et al. 2004; Wittke-Thompson et al. 2005; Luo et al. 2005, 2006; Balding 2006).

Three other studies analyzing *GSK3β* and schizophrenia have been published. Using a sample of 147 patients and 212 healthy individuals, Scassellati et al. (2004) analyzed two markers at positions −1727 A/T and −50 C/T and a (CAA) (*n*) repeat polymorphism localized in intron 1 of the gene. Their results showed no overall associations. However, in schizophrenia patients with paranoid subtype, they found that heterozygous (CAA) (three)/(CAA) (five) subjects were over-represented. Ikeda et al. (2005) analyzed seven markers and the (CAA) (*n*) repeat in 381 cases and 352 controls and detected no association with schizophrenia in a Japanese population. Lee et al. (2006) analyzed the same two markers in 138 cases and 350 controls and did not find any association in a Korean population. Furthermore, Szczepankiewicz et al. (2006) evaluated T50C in 432 cases and 408 controls and find no association with schizophrenia. Our results are the first analysis of genetic association the *GSK3β* gene with schizophrenia based on both matched case–control and family-based samples.

A number of human and animal studies have emerged that implicate GSK3 in the pathophysiology and genetics of schizophrenia (Cotter et al. 1998; Beaulieu et al. 2004; Emamian et al. 2004). In general, the data suggests that phosphorylation levels of GSK3β are reduced in schizo-

Table 3 Allelic and genotypic frequencies for the clozapine response association

SNP	Allele frequency						Genotype frequency						Association			
	Non-responder			Responder			Non-responder			Responder			Allele		Genotype	
		Samples	Percent		Samples	Percent		Samples	Percent		Samples	Percent	Chi square	*p* value	Chi square	*p* value
rs6805251	A	65	44.5	A	72	53.7	AA	14	19.2	AA	22	32.8	2.37	0.123	3.41	0.181
	C	81	55.5	C	62	46.3	AC	37	50.7	AC	28	41.8				
							CC	22	30.1	CC	17	25.4				
rs4688043	A	133	91.1	A	120	89.6	AA	61	83.6	AA	54	80.6	0.19	0.662	0.21	0.899
	G	13	8.9	G	14	10.4	AG	11	15.1	AG	12	17.9				
							GG	1	1.4	GG	1	1.5				
rs7624540	A	28	19.2	A	27	20.1	AA	2	2.7	AA	2	3.0	0.04	0.838	0.04	0.978
	G	118	80.8	G	107	79.9	AG	24	32.9	AG	23	31.3				
							GG	47	64.4	GG	42	62.7				
rs13319151	A	1	0.7	A	3	2.2	AA	0	0.0	AA	0	0.0	1.20	0.370	1.21	0.270
	G	145	99.3	G	131	97.8	AG	1	1.4	AG	3	4.5				
							GG	72	98.6	GG	64	95.5				
rs6438552	A	82	56.2	A	63	47.0	AA	22	30.1	AA	17	25.4	2.34	0.125	3.48	0.175
	G	64	43.8	G	71	53.0	AG	38	52.1	AG	29	43.3				
							GG	13	17.8	GG	21	31.3				
rs4072520	A	30	20.5	A	33	24.6	AA	2	2.7	AA	4	6.0	0.67	0.414	1.02	0.600
	C	116	79.5	C	101	75.4	AC	26	35.6	AC	25	37.3				
							CC	45	61.6	CC	38	56.7				
rs9878473	A	28	19.2	A	27	20.1	AA	22	30.1	AA	17	25.4	2.34	0.125	3.48	0.175
	T	118	80.8	T	107	79.9	AT	38	52.1	AT	29	43.3				
							TT	13	17.8	TT	21	31.3				
rs4491944	A	1	0.7	A	3	2.2	AA	0	0.0	AA	0	0.0	1.20	0.370	1.21	0.270
	T	145	99.3	T	131	97.8	AT	1	1.4	AT	3	4.5				
							TT	72	98.6	TT	64	95.5				
rs6772172	A	145	99.3	A	131	97.8	AA	72	98.6	AA	64	95.5	1.20	0.370	1.21	0.270
	G	1	0.07	G	1	2.2	AG	1	1.4	AG	3	4.5				
							GG	0	0.0	GG	0	0.0				
rs11919783	A	13	8.9	A	9	6.7	AA	0	0.0	AA	1	1.5	0.46	0.496	2.55	0.279
	G	133	91.1	G	125	93.3	AG	13	17.8	AG	7	10.4				
							GG	60	82.2	GG	59	88.1				
rs11923196	A	2	1.4	A	2	1.5	AA	0	0.0	AA	0	0.0	0.01	0.999	0.01	0.931
	T	144	98.6	T	132	98.5	AT	2	2.7	AT	2	3.0				
							TT	71	97.3	TT	65	97.1				
rs6779828	A	30	20.8	A	34	25.4	AA	2	2.8	AA	4	6.0	0.81	0.368	1.09	0.579
	G	114	79.2	G	100	74.6	AG	26	36.1	AG	26	38.8				
							GG	44	61.1	GG	37	55.2				
rs9846422	A	145	99.3	A	131	97.8	AA	72	98.6	AA	64	95.5	1.20	0.370	1.21	0.270
	G	1	0.07	G	1	2.2	AG	1	1.4	AG	3	4.5				
							GG	0	0.0	GG	0	0.0				
rs3755557	A	125	85.6	A	117	87.3	AA	53	72.6	AA	51	76.1	0.17	0.678	0.25	0.881
	C	21	14.4	C	17	12.7	AC	19	26.0	AC	15	22.4				
							CC	1	1.4	CC	1	1.5				

phrenia, resulting in increased GSK3β activity. If GSK3β regulation is altered in schizophrenia, it is possible that psychoactive compounds might also alter GSK3 signaling. Recent data suggest that antipsychotics have the opposite effect on Akt and GSK3 seen in schizophrenia, and this could be part of the mechanism of their therapeutic effects. In animal models, chronic antipsychotic treatment may increase the total levels of GSK3 (Alimohamad et al. 2005a, b) or Akt-induced serine phosphorylation of GSK3 (Alimohamad et al. 2005a; Emamian et al. 2004). Drugs that induce psychosis might also affect GSK3 signaling. Svenningsson et al. (2003) compared the short-term (15 min) effects of three psychomimetic drugs: amphetamine, D-lysergic acid (LSD; a serotonin agonist), and phencyclidine (PCP; a glutamate receptor antagonist) and found that in mice, all three increased pSer9-GSK3β in the frontal cortex and striatum but not in the hippocampus or cerebellum.

Recent evidence indicates that the expression and phosphorylation of GSK3 can be regulated by neurotransmitter receptors such as dopamine D2 and serotonin 5HT1/5HT2. Acute stimulation of dopamine D2 receptors elicits dephosphorylation and activation of GSK3 (Beaulieu et al. 2004, 2005). D2 blockade-mediated phosphorylation of GSK3 was accompanied with the activation of Akt, suggesting that Akt may be an upstream regulator of GSK3 in D2 receptor-mediated signaling (Beaulieu et al. 2007). On the other hand, the acute stimulation of serotonin 5HT1 receptors or blockade of 5HT2 receptors produces phosphorylation-induced inhibition of GSK3 in the rodent brain (Li et al. 2004). However, the mediators of the 5HT receptor-coupled regulation of GSK3 have not been well established; a recent report stated that they were not mediated by Akt (Li et al. 2007). The first report of a possible involvement of GSK3 in schizophrenia was by Yang et al. (1995), where they showed that both cellular activity and protein levels of kinase FA/GSK3α in the lymphocytes of schizophrenia patients were much lower than in normal controls.

We did not observe any association between *GSK3β* genetic variants and clozapine response. The finding that GSK3 is inhibited by both haloperidol and clozapine suggests that GSK3 may be a common target of both typical and atypical antipsychotics. In vivo results suggest that clozapine increases pSer9-GSK3β and pSer473-Akt (Kozlovsky et al. 2006; Roh et al. 2007), although Kang et al. (2004) did not find this effect in cultured cells SH-SY5Y that do not express D2 or 5HT2 receptors. Because of the links between GSK3 and both the pathophysiology of psychotic symptoms and antipsychotic medication effects (Jope and Roh 2006), further studies are required to understand how the associated markers reported here may affect these processes. Studies in larger, independent samples are also required to strengthen our findings.

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3.2 - Genetic association analysis of the GFR alpha genes with schizophrenia and clozapine response

Genetic association analysis of the GFR alpha genes with schizophrenia and clozapine response

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Abstract

GDNF (glial-cell-line derived neurotrophic factor) is a potent neurotrophic factor for dopaminergic neurons. Neuropsychiatric diseases and their treatments are associated with alterations in the levels of both GDNF and its receptor family (GDNF family receptor alpha or GFRA). *GFRA1*, *GFRA2* and *GFRA3* are located in chromosomal regions with suggestive linkage to schizophrenia. In this study we analyzed polymorphisms located in all four known GFRA genes and examined association with schizophrenia and clozapine response. We examined SNPs across the genes *GFRA1 - 4* in 219 matched case-control subjects, 85 small nuclear families and 140 schizophrenia patients taking clozapine for 6 months. *GFRA1* rs11197557 was associated with schizophrenia; *GFRA1 rs730357* and some haplotypes showed a significantly different transmission pattern, and two haplotypes (rs11197612-rs3781514 and rs12413585-rs730057-rs1197612) were associated with clozapine response. In *GFRA2,* three individual SNPs (rs1128397, rs13250096 and rs4567028) and several haplotypes showed association with response. *GFRA3* rs11242417 SNP and a haplotype containing all markers analyzed were associated with schizophrenia. None of the *GFRA4* markers evaluated had a significant association. We also found evidence for interactions between *GFRA1*, *2* and *3* associated with schizophrenia and clozapine response, consistent with the locations of these three genes within linkage regions for schizophrenia. *GFRA4*, which is not located in such a region, showed no interactions with the other genes in this regard. Our results presented nominally significant evidence that the GFRA genes may affect susceptibility to schizophrenia and response to clozapine treatment.

Keywords: schizophrenia, clozapine response. GDNF, GFR alpha, genetic association, family-based association test.

1 - Introduction

Schizophrenia is a serious, complex genetic neuropsychiatric disorder with a life-time prevalence of 0.5-1% in the population [1]. Family, twin and adoption studies convincingly demonstrate that relatives of affected individuals have a higher risk for schizophrenia and that this is largely the result of genetic factors [2]. A variety of different genes, each with small or moderate effect, are thought to be involved in the etiology of schizophrenia and the strongest findings to date include neuregulin-1, dysbindin and disrupted-in-schizophrenia-1 [3-6]. These genes share several important features: genetic association of specific SNPs and haplotypes with schizophrenia, chromosomal localization within linkage regions for schizophrenia and evidence for modulating neurodevelopment [7, 8].

It has been postulated that targeting the synthesis and secretion of neurotrophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and GDNF, might be a new approach to treating neurodegenerative and depressive disorders [9, 10], but this possibility has only recently been considered for antipsychotic drugs [11-13]. NGF and BDNF have been implicated in the neuroprotective actions of antipsychotic drugs [14, 15]. GDNF is a distantly-related member of the transforming growth factor-beta (TGF-beta) family that was isolated from a glial cell line [16]. Other members of the GDNF family were subsequently identified as neurturin, persephin and artemin. GDNF is synthesized in striatal cells and undergoes retrograde transport to dopaminergic cell bodies in the midbrain [17, 18]. GDNF enhances the survival of dopaminergic neurons [17, 19-21] and it has been postulated as the most potent neurotrophic factor for dopaminergic neurons [19].

GFRA proteins are non-signaling extracellular molecules that act as co-receptors for the binding of GDNF family proteins to the RET receptor. GFRAs are glycoproteins anchored to the cell surface by a C-terminal glycosylphosphatidylinositol-linkage [23]. Four GFRA family members have been recognized with similar structures and 30%–45% sequence identity. However each has a distinct expression pattern and affinity for GDNF-family ligands. GFRA1 has high affinity for GDNF and RET [24, 25], GFRA2 with neurturin [26, 27], GFRA3 with artemin [28] and GFRA4 with persephin [29, 30]. *GFRA1*, *GFRA2* and *GFRA3* are located within chromosomal regions that have been reported to be in linkage with schizophrenia [7].. Futhermore, TGF-beta genes are crucial regulators of neuron migration in *C. elegans* [31-33], a major component of cortical neurodevelopment that is hypothesized to be abnormal in schizophrenia. It has been suggested that GDNF plays a role in mammalian neuronal development [8, 34]. Based on the above rationale, we hypothesized that the GFRA genes may affect susceptibility to schizophrenia.

Variation in individual clinical response to antipsychotic treatment remains a critical problem in the management of serious mental illness. Treatment often proceeds by trial and error in order to determine the medication and dose that maximizes response and minimizes toxicity. Although a minority of patients may experience complete remission, a large proportion of patients continue to experience significant symptoms. In addition, a subset of patients develops drug-induced adverse events that range from troublesome to life-threatening. In spite of the wide array of medicines available, 10–20% of patients do not respond to treatment with an antipsychotic. An additional 20–30% who do respond early on eventually relapse and some develop serious side effects that cause them to discontinue medication [35, 36].

Some reports have shown GDNF system changes after administration of psychotropic drugs. Antidepressant and atypical antipsychotic drugs, but not typical drugs haloperidol, have been reported to increase GDNF release from C6 cells [37, 38]. GDNF levels are also altered by lithium and valproic acid administration [39, 40]. Furthermore, electroconvulsive seizure increased *GFRA1* and *2* mRNA levels in hippocampus; although *GDNF* and *c-ret* mRNAs were not significantly changed [41]. Given that this collection of psychiatric treatments affect components of the GDNF system, we hypothesized that genetic variation in the GFRA genes could affect response to clozapine treatment in schizophrenia.

2 - Methods

Clinical sample

All recruitment and clinical assessments were conducted with written informed consent, with the explicit approval of our institutional ethics review board and in accordance to Declaration of Helsinki. Clinical data and DNA samples were obtained from the probands of 85 small nuclear families, as well as 219 patients with a DSM-III-R or DSM-IV diagnosis of schizophrenia. The healthy controls (N=219) were matched for age (±5 years), sex, and ethnicity (146 male and 73 female cases and the same number of controls, mean age 36±8). The Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I) was administered by trained research assistants to each patient and diagnosis was supplemented by a review of medical records. The diagnosis was established via consensus procedures incorporating two of the investigators. The controls were screened for current or past history of major psychiatric disorders or substance misuse, and excluded if either was detected.

For the clozapine response sample, clinical data from 140 patients with a DSM-III-R or DSM-IV diagnosis of schizophrenia, almost all of whom were treatment refractory or intolerant of typical antipsychotic therapy [42], were obtained at the two following research clinics: Case Western Reserve University in Cleveland, OH (n=90) (63 males and 27 females in each group, mean age 36±8) and Hillside Hospital in Glen Oaks, NY (n=50) (33 males and 17 females in each group, mean age 35±8). After informed consent was obtained, patients underwent a washout period of 2 to 4 weeks during which, unless clinically necessary, they received no medications before starting clozapine. Clozapine treatment was continued for a minimum of 6 months during which patients were evaluated prospectively. Clozapine blood levels were monitored throughout the course of treatment to ascertain compliance. Treatment response was evaluated as the percentage score change on the 18 item Brief Psychiatric Rating Scale (BPRS). Treatment response was expressed as a dichotomous variable in the whole sample at 6 months using criteria based on those of Kane [42]: a reduction of ≥20% on the overall score of the BPRS from the baseline score at enrolment. There were no differences observed between the sites in terms of gender ratio, mean age, mean age of onset or response ratio. Caucasians and African-American subjects were not significantly different in terms of gender ratio, mean age, mean age of onset or response ratio (data not shown) [43].

Genetic analyses

Genomic DNA was extracted using the high salt method. We analyzed 26 SNPs in the *GFRA1* (rs1078080, rs11598215, rs3781514, rs2694783, rs2694801, rs3824840, rs12776813, rs7085306, rs10787627, rs9787429, rs12775655, rs11197557, rs10749189, rs11197567, rs3781539, rs7903297, rs17094340, rs7920934, rs10885877, rs4751956, rs11812459, rs10885888, rs12413585, rs730357, rs11197612), 17 in *GFRA2* (rs15881, rs4567027,

rs7813735, rs10088105, rs1128397, rs6988470, rs4237073, rs10283397, rs4739217, rs6587002, rs7014143, rs4567028, rs4739286, rs11993990, rs4078157, rs4739285, rs13250096), 4 in *GFRA3* (rs10036665, rs10952, rs11242417, rs7726580) and 2 in *GFRA4* (rs633924, rs6084432). Genotyping was performed by GoldenGate assay (Illumina, San Diego, CA, USA) at The Centre for Applied Genomics (TCAG) at the Hospital for Sick Children in Toronto, ON, Canada. Quality check procedure has been performed in this sample as described at [44].

Statistical analyses

Individual SNP analyses of clozapine responder (case) and non-responder (control) data and Hardy–Weinberg equilibrium assessment were performed using χ^2 tests. The statistical program used was the Statistical Package for the Social Sciences, version 10.0.7 for genotypic association and Haploview 4.0 [45] for allelic association. We applied the family-based association test (FBAT, version 1.0 [46]) under the assumption of an additive model, and PEDSTATS [47] for Hardy–Weinberg equilibrium in the family data. Linkage disequilibrium (LD) was assessed using Haploview, version 4.0. Haplotype analyses were performed using UNPHASED 3.0.10 [48], Haploview version 4.0 and FBAT. Gene–gene interactions were examined using the multifactor dimensionality reduction (MDR) method version 1.1.0 [49-51]. A detailed explanation of MDR has been published elsewhere [52].

3 - Results

Linkage disequilibrium analysis

Case-control sample: Pairwise LD between the SNPs is presented for each gene (Figure S1). In this study, we defined a haplotype block as a region over which < 5% of pairwise comparisons among informative SNPs showed strong evidence of historical recombination (upper confidence bound on D′ less than 0.9; [53]). Based on this definition we found 6 LD blocks in *GFRA1*, 5 in *GFRA2* and 1 block in each of *GFRA3* and *GFRA4*.

Family sample: Following the same criteria used for case-control sample, *GFRA1* showed 6 blocks, 3 blocks in *GFRA2*, and 1 block in each of *GFRA3* and *GFRA4* (FigureS2).

*Response sample: w*e observed 6 LD blocks in *GFRA1*, 4 in *GFRA2* and 1 block in *GFRA4* (FigureS3). No blocks were observed in *GFRA3*.

Single-marker analysis

Case-control sample: Significant deviations from Hardy–Weinberg equilibrium were observed for *GFRA1* rs3824840 (p = 0.043), *GFRA1* rs9787429 (p = 0.032), *GFRA1* rs10749189 (p = 0.030) and *GFRA2* rs4739217 (p = 0.008) in the control group. The following markers deviated from the Hardy-Weinberg equilibrium in cases: *GFRA1* rs1078080 (p = 0.016), *GFRA1* rs10885888 (p = 0.004), *GFRA4* rs633924 (p = 0.007) and *GFRA4* rs6084432 (p = 0.041). Only one SNP in the controls and two in the cases showed deviation from Hardy-Weinberg equilibrium at a significance threshold of p<0.01. The cases and controls were compared for genotype and allele frequencies across the markers (see Table S1 and Figure 3). In our population, no significant differences in genotype or allele frequency were found between cases and controls for any SNPs in *GFRA2* and *GFRA4*. *GFRA1* rs11197557 (allele p = 0.020, X^2 = 5.34; genotype p = 0.017, X^2 = 8.09) and *GFRA3* rs11242417 (allele p = 0.010, X^2 = 6.56;

genotype p = 0.014, X^2 = 8.50) were associated with schizophrenia. All markers that showed significant association were in Hardy-Weinberg equilibrium.

Family sample: Allele frequencies are presented in Table S2. *GFRA1 rs730357* A allele was overtransmitted to patients with schizophrenia (allele frequency = 0.742, z = 2.112, p = 0.035).

Response sample: Significant deviation from Hardy–Weinberg equilibrium was observed for *GFRA2* rs4739217 (p = 0.008) in the non-responder group. The following markers deviated significantly from Hardy-Weinberg equilibrium in the responder group: *GFRA1* rs3824840 (p = 0.030), *GFRA4* rs6084432 (p = 0.041) and *GFRA4* rs633924 (p = 0.007). Responder/non-responder groups were compared for genotype and allele frequencies across the markers (see Table S3 and Figure 4). In our population, no significant differences in genotype or allele frequency were seen between responders and non-responders for any SNPs in *GFRA1*, *GFRA3* and *GFRA4*. The following *GFRA2* SNPs showed significant association with treatment response: rs1128397 (allele p = 0.009, X^2 = 6.70; genotype p = 0.022, X^2 = 7.59); rs13250096 (allele p = 0.019, X^2 = 5.50; genotype p = 0.064, X^2 = 5.51) and rs4567028 (allele p = 0.047, X^2 = 3.92; genotype p = 0.068, X^2 = 5.39). All markers that showed significant association were in Hardy-Weinberg equilibrium.

Haplotype analysis

Case-control sample: Cases and controls were compared for haplotype frequencies across the GFRA markers. A small marker size (two and three markers) was chosen since high levels of haplotype diversity were expected due to the moderate LD observed in our samples [54]. *GFRA1*, *GFRA2* and *GFRA4* genes did not show any significant haplotypic association with two or three marker windows or haplotypes located in the same LD block. Considering haplotypes in the same LD block, the haplotype within *GFRA3* block 1 (rs10036665, rs10952, rs11242417 and rs7726580) T-T-C-G showed significant association with schizophrenia (control frequency = 0.173, case frequency = 0.109, p = 0.006; X^2 = 7.33; after 1 000 permutations p = 0.027).

Family sample: Analyzing haplotypes composed of *GFRA1* SNPs that reached lowest p-values (rs1078080 p = 0.113; rs7920934 p = 0.053 and rs730357 p = 0.035) the rs1078080-rs7920934 A-A haplotype was overtransmitted (frequency = 0.677, z = 2.166, p = 0.030; after 1 000 permutations p = 0.111); rs7920934-rs730357 G-G (frequency = 0.119, z = -2.361, p = 0.018; after 1,000 permutations p = 0.043) and rs1078080-rs7920934 G-G (frequency = 0.045, z = -2.614, p = 0.008; after 1,000 permutations p = 0.026) were undertransmitted. Considering haplotypes in the same LD block, inside *GFRA1* block 5 (rs7920934, rs10885877 and rs4751956) the G-G-G haplotype was undertransmitted to patients with schizophrenia (frequency = 0.223, transmitted: untransmitted ratio = 9 : 21, p = 0.028, X^2 = 4.80; after 1,000 permutations p = 0.290). In addition, in *GFRA1* block 6 (rs12413585, rs730357 and rs11197612) the G-A-G (frequency = 0.258; transmitted: untransmitted ratio = 23 : 11, p = 0.028, X^2 = 4.82, after 1,000 permutations p = 0.284) and G-G-G haplotypes showed significant different transmission pattern (frequency = 0.247, transmitted: untransmitted ratio = 9 : 22, p = 0.019, X^2 = 5.45; after 1 000 permutations p = 0.186). *GFRA2*, *GFRA3* and *GFRA4* genes did not show any significant haplotypic association with two or three marker windows or haplotypes located in the same LD block.

Response sample: Clozapine responders and non-responders were compared for haplotype frequencies across the GFRA markers. *GFRA1* markers showed significant haplotypic

association for rs11197612-rs3781514 (global p= 0.044, X^2 = 8.09). Considering haplotypes in the same LD block, inside *GFRA1* block 6 (rs12413585, rs730057 and rs1197612) the haplotype G-A-G showed association (non-responder frequency = 0.208, responder frequency = 0.330, p = 0.021, X^2 = 5.30; after 1,000 permutations p = 0.289). Two window *GFRA2* marker analysis found associations, the strongest of which consisted of rs1128397 and rs13250096 (global p = 0.005, X^2 = 12.6). The A-C (non-responder frequency = 0.073, responder frequency = 0.010, p = 0.0002, X^2 = 13.66) and T-G (non-responder frequency = 0.073, responder frequency = 0.010, p = 0.0002, X^2 = 13.66) haplotypes were associated with better response in our sample. The strongest *GFRA2* three-maker haplotype association was with rs1128397, rs4567028 and rs13250096 (global p = 0.012, X^2 = 16.2). The A-A-C (non-responder frequency = 0.070, responder frequency = 0.011, p = 0.001, X^2 = 10.55) and T-A-G (non-responder frequency = 0.348, responder frequency = 0.554, p = 0.0006, X^2 = 11.59) haplotypes were associated with better response in our sample. Considering haplotypes in the same LD block, the *GFRA2* block 1 (rs15881 and rs1128397) haplotype G-C showed association (non-responder frequency = 0.473, responder frequency = 0.321, p = 0.009, X^2 = 6.70; after 1 000 permutations p = 0.093). No haplotypic association was found in the *GFRA3* and *GFRA4*.

Gene-gene interactions

Case-control sample: Multi dimension reduction (MDR) analyses showed a significant association between combinations of the GFRA genes and schizophrenia. The best two-locus model contained *GFRA1* rs11197557 and *GFRA3* rs11242417 with a maximum cross-validation (CV) consistency of 10/10 and a maximum prediction accuracy of 56.6% (p = 0.001; after 1 000 permutations p = 0.377). The best three-locus model contained *GFRA1* rs11197557, *GFRA1* rs1078080 and *GFRA3* rs11242417, with a maximum CV consistency of 10/10 and a maximum prediction accuracy of 58.0% (p = 0.001; after 1 000 permutations p = 0.054). None of the interactions showed synergy (Figure S2).

Response sample: There were significant associations between gene-gene interactions and treatment response. The best two-locus model contained *GFRA1* rs1078080 and *GFRA2* rs15881 with a maximum cross-validation (CV) consistency of 4/10 and a maximum prediction accuracy of 46.6% (p = 0.37; after 1 000 permutations p = 0.623). The best three-locus model contained *GFRA1* rs10885888, *GFRA2* rs4237073 and *GFRA3* rs7726580, with a maximum CV consistency of 10/10 and a maximum prediction accuracy of 71.7% (p = 0.001; after 1 000 permutations p = 0.054). Likewise, the interaction dendrogram (Figure S2) placed *GFRA1* rs10885888, *GFRA2* rs4237073 and *GFRA3* rs7726580 on the same branch. Their position in the diagram indicates that this is the strongest interaction, which is consistent with location of these three genes within suggestive chromosomal linkage regions for schizophrenia. *GFRA4* is not located in a schizophrenia linkage region, and no interaction with the other genes was detected in this analysis.

4 - Discussion

This exploratory study examined the association of 26 SNPs in *GFRA1*, 17 in *GFRA2*, 4 in *GFRA3* and 2 in *GFRA4* and two phenotypes: diagnosis of schizophrenia, and clozapine response in schizophrenia patients. In the case-control analyses, the *GFRA1* rs11197557 and *GFRA3* rs11242417 markers showed nominally significant association with schizophrenia. One haplotype including *GFRA3* markers (rs10036665, rs10952, rs11242417, rs7726580) also showed association with schizophrenia. In the family-based sample, *GFRA1* gene showed allelic

and haplotypic association with schizophrenia. The rs730357 A allele; rs1078080-rs7920934 A-A and rs12413585-rs730357 -rs11197612 G-A-G haplotypes were overtransmitted. Haplotypes rs7920934-rs730357 G-G; rs1078080-rs7920934 G-G; rs7920934-rs10885877-rs4751956 G-G-G and rs12413585-rs730357 -rs11197612 G-G-G were undertransmitted. In the treatment response sample, *GFRA1* showed two protective haplotypes: rs11197612-rs3781514 and rs12413585-rs730057-rs1197612. *GFRA2* gene showed association with three individual SNPs (rs1128397, rs13250096 and rs4567028) and treatment response. Interestingly these polymorphisms did not show LD in the Haploview analysis, but some haplotypes showed association with treatment response, in particular a haplotype located in the first LD block (rs15881 and rs1128397). In *GFRA3* and *GFRA4* no genetic associations were observed.

All SNPs that presented allelic, genotypic or haplotypic nominal significant association were in Hardy-Weinberg equilibrium. We performed Bonferroni correction and permutation analysis (n = 1 000) for multiple testing for all of our individual SNP associations. After applying thresholds created after these corrections to our single SNP association findings, none remained significant (Bonferroni corrected $p<0.001$). Dealing with multiple testing is a controversial issue and has been intensely debated [55-57]. Considering the exploratory nature of this study, without prespecified hypotheses for most of our SNPs, there is no clear structure in the multiple tests [56]. "Significant" findings are therefore labeled as "exploratory" with confirmatory studies needed. Suggestion of potential gene–gene interaction was observed three times in analyses using non-parametric statistical models. The recently developed MDR method improves power by data reduction to efficiently identify potential gene–gene interactions in relatively small samples [49]. After cross-validation and permutation testing procedures were performed we found a trend ($p = 0.054$) for the interaction within *GFRA1* rs11197557, *GFRA1* rs1078080 and *GFRA3* rs11242417 in the case-control sample and *GFRA1* rs10885888, *GFRA2* rs4237073 and *GFRA3* rs7726580 in the response sample. *GFRA1* rs1078080, used in our best three-locus model in case-control sample, deviated from Hardy-Weinberg equilibrium in cases ($p = 0.016$) at a significance threshold of $p<0.05$. Trikalinos et al (2006) concluded that Hardy-Weinberg equilibrium should be routinely and transparently assessed in gene-disease association studies [58]. Discrepant results in these analyzes do not necessarily mean that postulated association should be dismissed, but they point to the need for more evidence and validation [58], especially considering that deviations can be a symptom of disease association, the implications of which are often under-exploited [59-61].

Of considerable interest, all significant findings were with markers in *GFRA1*, *GFRA2* and *GFRA3* that are located in chromosomal linkage regions for schizophrenia, while no significant results were found for *GFRA4*, which is not located in a linkage region. The interpretation of genetic linkage results is controversial and some degree of subjectivity enters into the determination of which regions of the genome should be considered to have truly significant evidence for linkage to schizophrenia. Two recent meta-analyses have summarized these findings [62, 63]. Most of the identified regions of linkage did not overlap across the two studies; however a region on chromosome 8p where *GFRA2* is located was supported by both investigations. The putative susceptibility genes for which the most follow-up genetic association data are available are those encoding dysbindin, neuregulin 1, D-amino-acid oxidase, D-amino-acid oxidase activator (formerly known as G72) and regulator of G-protein signalling 4 [64, 65]. Many of these candidate genes share a putative role in neurodevelopment, as does the GDNF pathway. The neurodevelopmental hypothesis for schizophrenia has strong support from the

effects of prenatal and perinatal insults, premorbid cognitive and neurological abnormalities, and the nature of histopathological abnormalities in brain tissue from schizophrenia patients [66].

GDNF was initially described as a trophic factor for dopaminergic neurons. Although considered one of the most potent neurotrophic factors for these neurons, GDNF is widely expressed throughout the brain, and exerts neuroprotective effects in several central and peripheral neuronal populations. Changes in the expression of other classes of neurotrophic factors and their receptors have been reported as a consequence of increased neural activity, injury and degeneration. Similarly, the expression of members of the GDNF family and their receptors are also affected by neural insults and degeneration. Studies have shown that expression of GFRA and their ligands can be altered in the rodent following peripheral nerve injury, ischemia and seizures [67]. Dopamine D2 receptor (DRD2) null mutant mice have altered GDNF levels, although *GFRA1* mRNA expression was unchanged [68]. Moreover, $GDNF^{+/-}$ mutant mice have abnormal hippocampal synaptic transmission and impaired spatial learning [69, 70]. Rosa et al (2006) reported increased serum GDNF levels in bipolar patients during acute manic and depressive episodes when compared with matched healthy controls [71]. Medication treatments used for psychiatric disorders also alter GFRA receptors and ligand levels [37-39, 41]. Taken together, these findings suggest that alterations in GDNF signalling may play a role in neuropsychiatric disease and associated treatment effects. Our results, in combination with these other observations, suggest that the GFRA receptors may be involved in a pathway that affects neurodevelopment in schizophrenia, but further work is clearly required to strengthen this hypothesis.

Disclosure/Conflicts of interest

Souza, Romano-Silva, Culotti, MacNeil and Wong have nothing to declare. Lieberman has served as a consultant/ advisor or grantee of Acadia, Astra Zeneca, Bristol-Myers Squibb, Eli Lilly, GlaxoSmithKline, Janssen Pharmaceutica, Lundbeck, Merck, Organon, Pfizer and Wyeth; and holds a patent from Repligen. Meltzer declares that he is a consultant or grantee of Abbott, Acadia, ARYx, Astra Zeneca, Bristol Myers Squibb, Eli Lilly, Janssen, Memory, Minster, Organon, Pfizer, Solvay, Wyeth, and Vanda. Kennedy declares that he is a consultant for GlaxoSmithKline.

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Table 1:

rs		Control			Case	
		n	%		n	%
GFRA1 rs1078080	A	271	61.9	A	271	64.2
	G	167	38.1	G	151	35.8
	AA	87	39.7	AA	95	45.0
	AG	97	44.3	AG	81	38.4
	GG	35	16.0	GG	35	16.6
GFRA1 rs2694801	A	28	19.2	A	19	14.2
	G	118	80.8	G	115	85.8
	AA	04	05.5	AA	01	01.5
	AG	20	27.4	AG	17	25.4
	GG	49	67.1	GG	49	73.1
GFRA1 rs10787627	A	202	46.1	A	195	46.2
	G	236	53.9	G	227	53.8
	AA	43	19.6	AA	45	21.3
	AG	116	53.0	AG	105	49.8
	GG	60	27.0	GG	61	27.4
GFRA1 rs10749189	A	269	61.4	A	272	64.5
	G	169	38.6	G	150	35.5
	AA	75	34.2	AA	85	40.3
	AG	119	54.3	AG	102	48.3
	GG	25	11.4	GG	24	11.4
GFRA1 rs17094340	A	358	81.7	A	363	86.0
	G	80	18.3	G	59	14.0
	AA	147	67.1	AA	153	72.5
	AG	64	29.2	AG	57	27.0
	GG	08	03.7	GG	01	00.5
GFRA1 rs11812459	A	175	40.1	A	191	45.3
	G	261	59.9	G	231	54.7
	AA	35	16.1	AA	46	21.8
	AG	105	48.2	AG	99	46.9
	GG	78	35.8	GG	66	31.3

rs		Control			Case	
		n	%		n	%
GFRA1 rs11598215	A	105	24.0	A	86	20.4
	G	333	76.0	G	336	79.6
	AA	14	06.4	AA	09	04.3
	AG	77	35.2	AG	68	32.2
	GG	128	58.4	GG	134	63.5
GFRA1 rs3824840	A	108	24.9	A	100	23.7
	G	326	75.1	G	322	76.3
	AA	19	08.8	AA	09	04.3
	AG	70	32.3	AG	82	38.9
	GG	128	59.0	GG	120	56.9
GFRA1 rs9787429	A	95	21.9	A	87	20.6
	G	339	78.1	G	335	79.4
	AA	05	02.3	AA	06	02.8
	AG	85	39.2	AG	75	35.5
	GG	127	58.5	GG	130	61.6
GFRA1 rs11197567	A	102	23.3	A	115	27.3
	G	336	76.7	G	307	72.7
	AA	15	6.8	AA	15	7.1
	AG	72	32.9	AG	85	40.3
	GG	132	60.3	GG	111	52.6
GFRA1 rs7920934	A	328	74.9	A	318	75.7
	G	110	25.1	G	102	24.3
	AA	123	56.2	AA	122	58.1
	AG	82	37.4	AG	74	35.2
	GG	14	06.4	GG	14	06.7
GFRA1 rs10885888	A	97	22.4	A	92	21.9
	G	337	77.6	G	328	78.1
	AA	10	04.6	AA	03	01.4
	AG	77	35.2	AG	86	40.8
	GG	130	59.4	GG	121	59.4

rs		Control			Case	
		n	%		n	%
GFRA1 rs3781514	A	70	16.0	A	69	16.4
	G	368	84.0	G	353	83.6
	AA	04	01.8	AA	07	03.3
	AG	62	28.3	AG	55	26.1
	GG	153	69.9	GG	149	70.6
GFRA1 rs12776813	A	67	15.3	A	67	15.9
	G	371	84.7	G	355	84.1
	AA	05	02.3	AA	04	01.9
	AG	57	26.0	AG	59	28.0
	GG	157	71.7	GG	148	70.1
GFRA1 rs12775655	A	324	74.0	A	330	78.2
	C	114	26.0	C	92	21.8
	AA	119	54.3	AA	128	60.7
	AC	86	39.3	AC	74	35.1
	CC	14	06.4	CC	09	04.3
GFRA1 rs3781539	A	375	85.6	A	358	84.8
	G	63	14.4	G	64	15.2
	AA	161	73.5	AA	152	72.0
	AG	53	24.2	AG	54	25.6
	GG	05	02.3	GG	05	02.4
GFRA1 rs10885877	A	106	24.2	A	115	27.3
	G	332	75.8	G	307	72.7
	AA	14	06.4	AA	13	06.2
	AG	78	35.6	AG	89	42.2
	GG	127	58.0	GG	109	51.7
GFRA1 rs12413585	A	226	51.6	A	210	49.8
	G	212	48.4	G	212	50.2
	AA	57	26.0	AA	47	22.3
	AG	112	51.1	AG	116	55.0
	GG	50	22.8	GG	48	22.7

rs		Control			Case	
		n	%		n	%
GFRA1 rs2694783	A	250	57.1	A	247	58.5
	T	188	42.9	T	175	41.5
	AA	68	31.1	AA	70	33.2
	AT	114	52.1	AT	107	50.7
	TT	37	16.9	TT	34	16.1
GFRA1 rs7085306	A	263	60.0	A	255	60.4
	G	175	40.0	G	167	39.6
	AA	80	36.5	AA	71	33.6
	AG	103	47.0	AG	113	53.6
	GG	36	16.4	GG	27	12.8
GFRA1 rs11197557	A	99	22.6	A	69	16.4
	G	339	77.4	G	353	83.6
	AA	04	01.8	AA	00	0.00
	AG	91	41.6	AG	69	32.7
	GG	124	56.6	GG	142	67.3
GFRA1 rs7903297	A	205	46.3	A	188	44.5
	C	235	53.7	C	234	55.5
	AA	50	22.8	AA	41	19.4
	AC	103	47.0	AC	106	50.2
	CC	66	30.1	CC	64	30.3
GFRA1 rs4751956	A	203	46.3	A	185	43.8
	G	235	53.7	G	237	56.2
	AA	49	22.4	AA	40	19.0
	AG	105	47.9	AG	105	49.8
	GG	65	29.7	GG	66	31.3
GFRA1 rs730357	A	312	71.2	A	312	73.9
	G	126	28.8	G	110	26.1
	AA	112	51.1	AA	115	54.5
	AG	88	40.2	AG	82	38.9
	GG	19	8.7	GG	14	6.6

rs		Control			Case	
		n	%		n	%
GFRA1 rs1197612	A	45	30.8	A	38	28.4
	G	101	69.2	G	96	71.6
	AA	09	12.3	AA	07	10.4
	AG	27	37.0	AG	24	35.8
	GG	37	50.7	GG	36	53.7
GFRA2 rs7813735	A	76	52.1	A	73	54.5
	G	70	47.9	G	61	45.5
	AA	18	24.7	AA	20	29.9
	AG	40	54.8	AG	33	49.3
	GG	15	20.5	GG	14	20.9
GFRA2 rs4078157	A	61	41.8	A	47	35.1
	G	85	58.2	G	87	64.9
	AA	13	17.8	AA	8	11.9
	AG	35	47.9	AG	31	46.3
	GG	25	34.2	GG	28	41.8
GFRA2 rs4567028	A	104	71.2	A	109	81.3
	G	42	28.8	G	25	18.7
	AA	35	47.9	AA	45	67.2
	AG	34	46.6	AG	19	28.4
	GG	04	05.5	GG	03	04.5
GFRA2 rs1128397	A	69	47.3	A	43	32.1
	T	77	52.7	T	91	67.9
	AA	14	19.2	AA	7	10.4
	AT	41	56.2	AT	29	43.3
	TT	18	24.7	TT	31	46.3
GFRA3 rs11242417	A	124	84.9	A	113	84.3
	C	22	15.1	C	21	15.7
	AA	52	71.2	AA	48	71.6
	AC	20	27.4	AC	17	25.4
	CC	01	01.4	CC	02	03.0

rs		Control			Case	
		n	%		n	%
GFRA2 rs15881	A	62	42.5	A	72	53.7
	C	84	57.5	C	62	46.3
	AA	12	16.4	AA	21	31.3
	AC	38	52.1	AC	30	44.8
	CC	23	31.5	CC	16	23.9
GFRA2 rs10088105	A	22	15.1	A	13	09.7
	G	124	84.9	G	121	90.3
	AA	02	02.7	AA	02	03.0
	AG	18	24.7	AG	09	13.4
	GG	53	72.6	GG	56	83.6
GFRA2 rs13250096	C	18	12.3	C	06	4.5
	G	128	87.7	G	128	95.5
	CC	01	01.4	CC	00	0.0
	CG	16	21.9	CG	06	9.0
	GG	56	76.7	GG	61	91.0
GFRA2 rs6587002	A	99	67.8	A	91	67.9
	G	47	32.2	G	43	32.1
	AA	36	49.3	AA	33	49.3
	AG	27	37.0	AG	25	37.3
	GG	10	13.7	GG	09	13.4
GFRA2 rs6988470	A	77	52.7	A	70	52.2
	G	69	47.3	G	64	47.8
	AA	20	27.4	AA	19	28.4
	AG	37	50.7	AG	32	47.8
	GG	16	21.9	GG	16	23.9
GFRA3 rs7726580	A	50	34.2	A	45	33.6
	G	96	65.8	G	89	66.4
	AA	8	11.0	AA	10	14.9
	AG	34	46.6	AG	25	37.3
	GG	31	42.5	GG	32	47.8

rs		Control			Case	
		n	%		n	%
GFRA2 rs10283397	C	66	45.2	C	62	46.3
	G	80	54.8	G	72	53.7
	CC	14	19.2	CC	17	25.4
	CG	38	52.1	CG	28	41.8
	GG	21	28.8	GG	22	32.8
GFRA2 rs4567027	C	104	71.2	C	102	76.1
	G	42	28.8	G	32	23.9
	CC	36	49.3	CC	39	58.2
	CG	32	43.8	CG	24	35.8
	GG	05	06.8	GG	04	06.0
GFRA2 rs4739285	A	85	58.2	A	64	47.8
	G	61	41.8	G	70	52.2
	AA	24	32.9	AA	19	28.4
	AG	37	50.7	AG	26	38.8
	GG	12	16.4	GG	22	32.8
GFRA2 rs4739217	C	79	55.6	C	62	46.3
	G	63	44.4	G	72	53.7
	CC	24	32.9	CC	19	28.4
	CG	31	42.5	CG	24	35.8
	GG	16	21.9	GG	24	35.8
GFRA3 rs10036665	A	117	80.1	A	105	78.4
	T	29	19.9	T	29	21.6
	AA	47	64.4	AA	41	61.2
	AT	23	31.5	AT	23	34.3
	TT	03	04.1	TT	03	04.5
GFRA4 rs6084432	A	23	15.8	A	23	17.2
	G	123	84.2	G	111	82.8
	AA	01	01.4	AA	02	03.0
	AG	21	28.8	AG	19	28.4
	GG	51	69.9	GG	46	68.7

rs		Control			Case	
		n	%		n	%
GFRA2 rs11993990	A	71	48.6	A	67	50.0
	G	75	51.4	G	67	50.0
	AA	15	20.5	AA	20	29.9
	AG	41	56.2	AG	27	40.3
	GG	17	23.3	GG	20	29.9
GFRA2 rs4237073	A	62	42.5	A	71	53.0
	G	42	57.5	G	63	47.0
	AA	14	19.2	AA	20	29.9
	AG	34	46.6	AG	31	46.3
	GG	25	34.2	GG	16	23.9
GFRA2 rs4739286	A	43	29.5	A	33	24.6
	G	103	70.5	G	101	75.4
	AA	04	05.5	AA	06	09.0
	AG	35	47.9	AG	21	33.3
	GG	34	46.6	GG	40	59.7
GFRA2 rs7014143	A	53	36.3	A	50	37.3
	C	93	63.7	C	84	62.7
	AA	9	12.3	AA	10	14.9
	AC	35	47.9	AC	30	44.8
	CC	29	39.7	CC	27	40.3
GFRA3 rs10952	A	43	29.5	A	36	26.9
	T	103	70.5	T	98	73.1
	AA	06	08.2	AA	05	07.5
	AT	31	42.5	AT	26	38.8
	TT	36	49.3	TT	36	53.7
GFRA4 rs633924	A	89	61.0	A	79	59.0
	G	87	39.0	G	55	41.0
	AA	27	37.0	AA	24	35.8
	AG	35	47.9	AG	31	46.3
	GG	11	15.1	GG	12	17.9

Table 2

Gene	rs	Allele	Frequency	Family	S	E(S)	Var(S)	Z	P
GFRA1	rs1078080	A	0.696	32	48	42.500	12.028	1.586	0.113
	rs11598215	A	0.190	25	13	17.000	8.778	-1.350	0.177
	rs3781514	A	0.198	30	18	17.733	8.462	0.092	0.927
	rs2694783	A	0.564	41	41	44.900	14.712	-1.017	0.309
	rs2694801	A	0.233	27	22	20.833	8.194	0.408	0.684
	rs3824840	A	0.281	29	25	23.433	7.934	0.556	0.578
	rs12776813	A	0.183	30	15	17.333	8.222	-0.814	0.416
	rs7085306	A	0.573	40	46	45.067	13.240	0.257	0.798
	rs10787627	A	0.461	40	40	39.600	13.462	0.109	0.913
	rs9787429	A	0.248	24	21	21.833	7.194	-0.311	0.756
	rs12775655	A	0.772	32	45	44.100	9.212	0.297	0.767
	rs11197557	A	0.216	33	19	19.833	9.417	-0.272	0.786
	rs10749189	A	0.617	40	48	45.900	13.712	0.567	0.571
	rs11197567	A	0.273	26	20	20.633	8.354	-0.219	0.827
	rs3781539	A	0.818	21	32	29.867	6.104	0.863	0.388
	rs7903297	A	0.412	32	34	31.833	11.417	0.641	0.521
	rs17094340	A	0.845	20	28	29.833	5.194	-0.804	0.421
	rs7920934	A	0.764	34	53	46.667	10.722	1.934	0.053
	rs10885877	A	0.311	25	25	21.333	7.667	1.324	0.185
	rs4751956	A	0.472	35	39	35.333	12.167	1.051	0.293
	rs11812459	A	0.450	29	30	30.667	10.953	-0.201	0.840
	rs10885888	A	0.241	26	14	16.583	7.632	-0.935	0.350
	rs12413585	A	0.499	35	34	33.333	12.444	0.189	0.850
	rs730357	A	0.742	30	45	38.167	10.472	2.112	0.035
	rs11197612	A	0.388	30	25	25.333	10.167	-0.105	0.917
GFRA2	rs15881	A	0.500	29	32	31.000	10.614	0.307	0.759
	rs4567027	C	0.713	33	47	44.667	10.558	0.718	0.473
	rs7813735	A	0.485	33	29	31.333	12.558	-0.658	0.510
	rs10088105	A	0.151	18	09	10.333	5.222	-0.583	0.560
	rs1128397	A	0.432	37	29	33.167	13.086	-1.152	0.249
	rs6988470	A	0.548	30	32	31.167	10.972	0.252	0.801
	rs4237073	A	0.519	37	43	39.700	12.632	0.928	0.353
	rs4739217	C	0.483	33	35	35.333	12.500	-0.094	0.925
	rs10283397	C	0.483	30	31	30.200	10.827	0.243	0.808
	rs6587002	A	0.662	27	34	33.833	8.972	0.056	0.956
	rs7014143	A	0.418	29	27	24.367	9.632	0.848	0.396
	rs4567028	A	0.777	29	45	42.500	9.528	0.810	0.418
	rs4739286	A	0.315	30	24	24.000	10.722	0.000	1.000
	rs11993990	A	0.554	37	49	46.667	12.953	0.648	0.517
	rs4078157	A	0.350	33	26	27.833	10.808	-0.558	0.577
	rs4739285	A	0.503	35	37	38.167	12.250	-0.333	0.739
	rs13250096	C	0.157	18	11	12.333	5.722	-0.557	0.577
GFRA3	rs10036665	A	0.830	16	28	25.167	4.972	1.271	0.204
	rs10952	A	0.365	34	25	24.167	10.250	0.260	0.795
	rs11242417	A	0.856	14	24	20.667	4.222	1.622	0.105
	rs7726580	A	0.413	36	26	27.667	11.336	-0.495	0.621
GFRA4	rs633924	A	0.647	33	45	42.167	11.472	0.837	0.403
	rs6084432	A	0.200	22	10	13.667	6.944	-1.391	0.164

Table 3

rs		Non-responder			Responder		rs		Non-responder			Responder		rs		Non-responder			Responder		rs		Non-responder			Responder	
		n	%		n	%			n	%		n	%			n	%		n	%			n	%		n	%
GFRA1 rs1078080	A	87	59.6	A	77	57.5	GFRA1 rs11598215		34	23.3	A	37	27.6	GFRA1 rs3781514	A	14	09.6	A	17	12.7	GFRA1 rs2694783		A	86	58.9	A	82	61.2
	G	59	40.4	G	57	42.5			112	76.7	G	97	72.4		G	132	90.4	G	117	87.3			T	60	41.1	T	52	38.8
	AA	28	38.4	AA	25	37.3			04	05.5	AA	08	11.9		AA	01	01.4	AA	01	01.5			AA	28	38.4	AA	26	38.8
	AG	31	42.5	AG	27	40.3			26	35.6	AG	21	31.3		AG	12	16.4	AG	15	22.4			AT	30	41.1	AT	30	44.8
	GG	14	19.2	GG	15	22.4			43	58.9	GG	38	56.7		GG	60	82.2	GG	51	82.2			TT	15	20.5	TT	11	16.4
GFRA1 rs2694801	A	28	19.2	A	19	14.2	GFRA1 rs3824840		31	21.2	A	28	20.9	GFRA1 rs12776813	A	21	14.4	A	15	11.2	GFRA1 rs7085306		A	93	63.7	A	89	66.4
	G	118	80.8	G	115	85.8			115	78.8	G	106	79.1		G	125	85.6	G	119	88.8			G	53	36.3	G	45	33.6
	AA	04	05.5	AA	01	01.5			03	4.1	AA	00	0.00		AA	01	01.4	AA	01	01.5			AA	30	41.1	AA	27	40.3
	AG	20	27.4	AG	17	25.4			25	34.2	AG	28	41.8		AG	19	26.0	AG	13	19.4			AG	33	45.2	AG	35	52.2
	GG	49	67.1	GG	49	73.1			45	61.6	GG	39	58.2		GG	53	72.6	GG	53	79.1			GG	10	13.7	GG	05	07.5
GFRA1 rs10787627	A	68	46.6	A	62	46.3	GFRA1 rs9787429		28	19.2	A	25	18.7	GFRA1 rs12775655	A	119	81.5	A	103	76.9	GFRA1 rs11197557		A	22	15.1	A	20	14.9
	G	78	53.4	G	72	53.7			118	80.8	G	109	81.3		C	27	18.5	G	31	23.1			G	124	84.9	G	114	85.1
	AA	20	27.4	AA	16	27.4			01	01.4	AA	00	0.00		AA	48	65.8	AA	40	59.7			AA	01	1.4	AA	00	0.00
	AG	28	38.4	AG	30	38.4			26	35.6	AG	25	37.3		AC	23	31.5	AG	23	34.3			AG	20	27.4	AG	20	29.9
	GG	25	34.2	GG	21	34.2			46	63.0	GG	42	62.7		CC	02	02.7	GG	04	06.0			GG	52	21.2	GG	47	70.1
GFRA1 rs10749189	A	102	69.9	A	82	61.2	GFRA1 rs11197567		32	21.9	A	35	26.1	GFRA1 rs3781539	A	131	89.7	A	120	89.6	GFRA1 rs7903297		A	59	40.4	A	57	42.5
	G	44	30.1	G	52	38.8			114	78.1	G	99	73.9		G	15	10.3	G	14	10.4			C	87	59.6	C	77	57.5
	AA	33	45.2	AA	24	35.8			02	02.7	AA	03	04.5		AA	59	80.8	AA	53	79.1			AA	08	11.0	AA	10	14.9
	AG	36	49.3	AG	34	50.7			28	38.4	AG	29	43.3		AG	13	17.8	AG	14	20.9			AC	43	58.9	AC	37	55.2
	GG	04	05.5	GG	09	13.4			43	58.9	GG	35	52.2		GG	01	1.4	GG	00	00.0			CC	22	30.1	CC	20	29.9
GFRA1 rs17094340	A	130	89.0	A	118	88.1	GFRA1 rs7920934		100	68.5	A	100	74.6	GFRA1 rs10885877	A	31	21.2	A	41	30.6	GFRA1 rs4751956		A	61	41.8	A	47	35.1
	G	16	11.0	G	16	11.9			46	31.5	G	34	25.4		G	115	78.8	G	93	69.4			G	85	58.2	G	87	64.9
	AA	57	78.1	AA	51	76.1			35	47.9	AA	39	58.2		AA	04	05.5	AA	05	07.5			AA	14	19.2	AA	11	16.4
	AG	16	21.9	AG	16	23.9			30	41.1	AG	22	32.8		AG	23	31.5	AG	31	46.3			AG	33	45.2	AG	25	37.3
	GG	00	00.0	GG	00	00.0			08	11.0	GG	06	09.0		GG	46	63.0	GG	31	46.3			GG	26	35.6	GG	31	46.3
GFRA1 rs11812459	A	64	43.8	A	65	48.5	GFRA1 rs10885888		45	30.8	A	33	24.6	GFRA1 rs12413585	A	77	52.7	A	60	44.8	GFRA1 rs730357		A	105	77.6	A	104	77.6
	G	82	56.2	G	69	51.5			101	69.2	G	101	75.4		G	69	47.3	G	74	55.2			G	41	22.4	G	30	22.4
	AA	13	17.8	AA	17	25.4			06	08.2	AA	04	06.0		AA	20	27.4	AA	13	19.4			AA	38	52.1	AA	39	58.2
	AG	38	52.1	AG	31	46.3			33	45.2	AG	25	37.3		AG	37	50.7	AG	34	50.7			AG	29	39.7	AG	26	38.8
	GG	22	30.1	GG	19	28.4			34	46.6	GG	38	56.7		GG	16	21.9	GG	20	29.9			GG	06	08.2	GG	02	03.0

rs	Non-responder			Responder		
		n	%		n	%
GFRA1 rs11197612	A	45	30.8	A	38	28.4
	G	101	69.2	G	96	71.6
	AA	09	12.3	AA	07	10.4
	AG	27	37.0	AG	24	35.8
	GG	37	50.7	GG	36	53.7
GFRA2 rs7813735	A	76	52.1	A	73	54.5
	G	70	47.9	G	61	45.5
	AA	18	24.7	AA	20	29.9
	AG	40	54.8	AG	33	49.3
	GG	15	20.5	GG	14	20.9
GFRA2 rs4078157	A	61	41.8	A	47	35.1
	G	85	58.2	G	87	64.9
	AA	13	17.8	AA	8	11.9
	AG	35	47.9	AG	31	46.3
	GG	25	34.2	GG	28	41.8
GFRA2 rs4567028	A	104	71.2	A	109	81.3
	G	42	28.8	G	25	18.7
	AA	35	47.9	AA	45	67.2
	AG	34	46.6	AG	19	28.4
	GG	04	05.5	GG	03	04.5
GFRA2 rs1128397	A	69	47.3	A	43	32.1
	T	77	52.7	T	91	67.9
	AA	14	19.2	AA	7	10.4
	AT	41	56.2	AT	29	43.3
	TT	18	24.7	TT	31	46.3
GFRA3 rs11242417	A	124	84.9	A	113	84.3
	C	22	15.1	C	21	15.7
	AA	52	71.2	AA	48	71.6
	AC	20	27.4	AC	17	25.4
	CC	01	01.4	CC	02	03.0

rs	Non-responder			Responder		
		n	%		n	%
GFRA2 rs15881	A	62	42.5	A	72	53.7
	C	84	57.5	C	62	46.3
	AA	12	16.4	AA	21	31.3
	AC	38	52.1	AC	30	44.8
	CC	23	31.5	CC	16	23.9
GFRA2 rs10088105	A	22	15.1	A	13	09.7
	G	124	84.9	G	121	90.3
	AA	02	02.7	AA	02	03.0
	AG	18	24.7	AG	09	13.4
	GG	53	72.6	GG	56	83.6
GFRA2 rs13250096	C	18	12.3	C	06	4.5
	G	128	87.7	G	128	95.5
	CC	01	01.4	CC	00	0.0
	CG	16	21.9	CG	06	9.0
	GG	56	76.7	GG	61	91.0
GFRA2 rs6587002	A	99	67.8	A	91	67.9
	G	47	32.2	G	43	32.1
	AA	36	49.3	AA	33	49.3
	AG	27	37.0	AG	25	37.3
	GG	10	13.7	GG	09	13.4
GFRA2 rs6988470	A	77	52.7	A	70	52.2
	G	69	47.3	G	64	47.8
	AA	20	27.4	AA	19	28.4
	AG	37	50.7	AG	32	47.8
	GG	16	21.9	GG	16	23.9
GFRA3 rs7726580	A	50	34.2	A	45	33.6
	G	96	65.8	G	89	66.4
	AA	8	11.0	AA	10	14.9
	AG	34	46.6	AG	25	37.3
	GG	31	42.5	GG	32	47.8

rs	Non-responder			Responder		
		n	%		n	%
GFRA2 rs10283397	C	66	45.2	C	62	46.3
	G	80	54.8	G	72	53.7
	CC	14	19.2	CC	17	25.4
	CG	38	52.1	CG	28	41.8
	GG	21	28.8	GG	22	32.8
GFRA2 rs4567027	C	104	71.2	C	102	76.1
	G	42	28.8	G	32	23.9
	CC	36	49.3	CC	39	58.2
	CG	32	43.8	CG	24	35.8
	GG	05	06.8	GG	04	06.0
GFRA2 rs4739285	A	85	58.2	A	64	47.8
	G	61	41.8	G	70	52.2
	AA	24	32.9	AA	19	28.4
	AG	37	50.7	AG	26	38.8
	GG	12	16.4	GG	22	32.8
GFRA2 rs4739217	C	79	55.6	C	62	46.3
	G	63	44.4	G	72	53.7
	CC	24	32.9	CC	19	28.4
	CG	31	42.5	CG	24	35.8
	GG	16	21.9	GG	24	35.8
GFRA3 rs10036665	A	117	80.1	A	105	78.4
	T	29	19.9	T	29	21.6
	AA	47	64.4	AA	41	61.2
	AT	23	31.5	AT	23	34.3
	TT	03	04.1	TT	03	04.5
GFRA4 rs6084432	A	23	15.8	A	23	17.2
	G	123	84.2	G	111	82.8
	AA	01	01.4	AA	02	03.0
	AG	21	28.8	AG	19	28.4
	GG	51	69.9	GG	46	68.7

rs	Non-responder			Responder		
		n	%		n	%
GFRA2 rs11993990	A	71	48.6	A	67	50.0
	G	75	51.4	G	67	50.0
	AA	15	20.5	AA	20	29.9
	AG	41	56.2	AG	27	40.3
	GG	17	23.3	GG	20	29.9
GFRA2 rs4237073	A	62	42.5	A	71	53.0
	G	42	57.5	G	63	47.0
	AA	14	19.2	AA	20	29.9
	AG	34	46.6	AG	31	46.3
	GG	25	34.2	GG	16	23.9
GFRA2 rs4739286	A	43	29.5	A	33	24.6
	G	103	70.5	G	101	75.4
	AA	04	05.5	AA	06	09.0
	AG	35	47.9	AG	21	33.3
	GG	34	46.6	GG	40	59.7
GFRA2 rs7014143	A	53	36.3	A	50	37.3
	C	93	63.7	C	84	62.7
	AA	9	12.3	AA	10	14.9
	AC	35	47.9	AC	30	44.8
	CC	29	39.7	CC	27	40.3
GFRA3 rs10952	A	43	29.5	A	36	26.9
	T	103	70.5	T	98	73.1
	AA	06	08.2	AA	05	07.5
	AT	31	42.5	AT	26	38.8
	TT	36	49.3	TT	36	53.7
GFRA4 rs633924	A	89	61.0	A	79	59.0
	G	87	39.0	G	55	41.0
	AA	27	37.0	AA	24	35.8
	AG	35	47.9	AG	31	46.3
	GG	11	15.1	GG	12	17.9

Figure 1

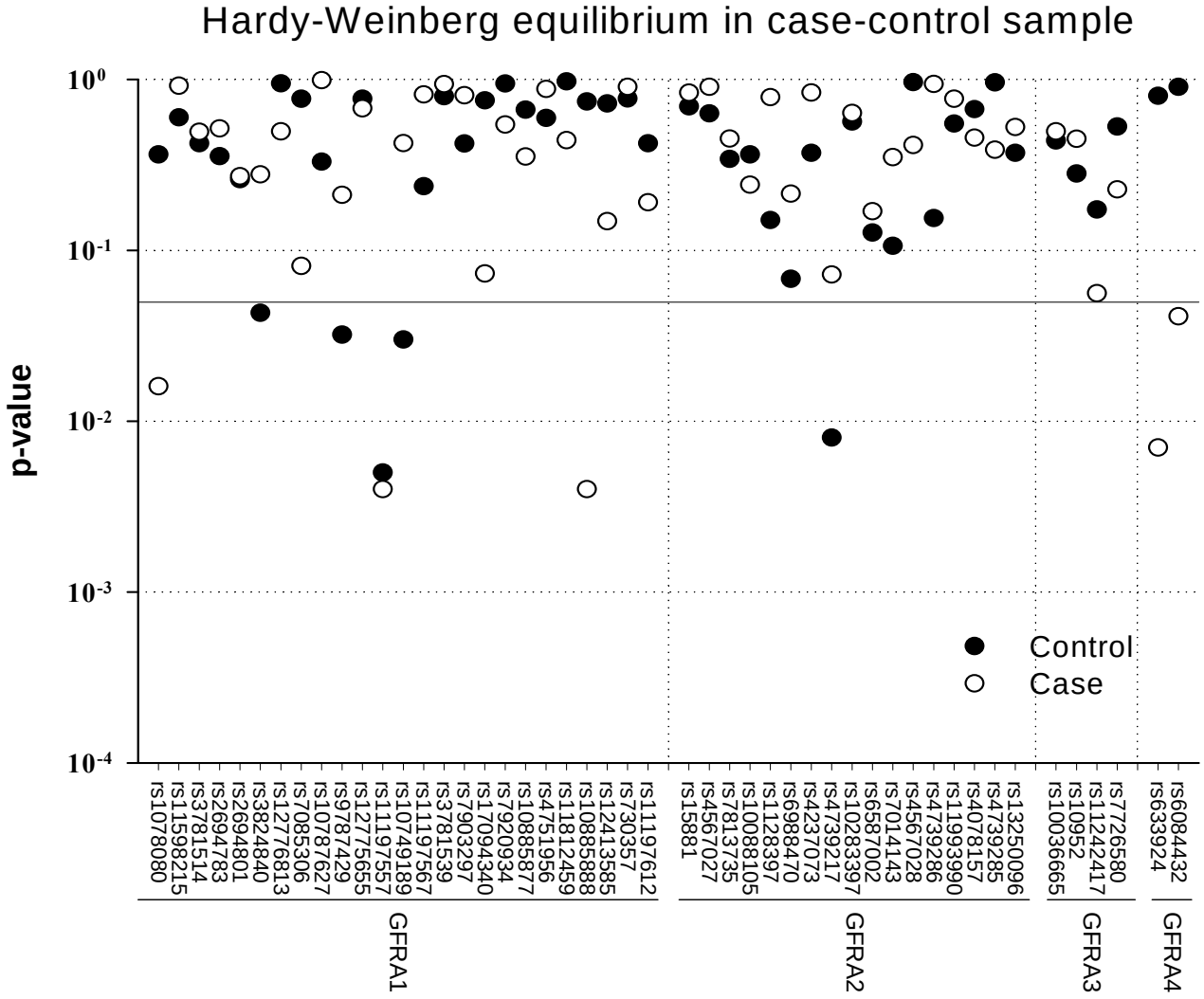
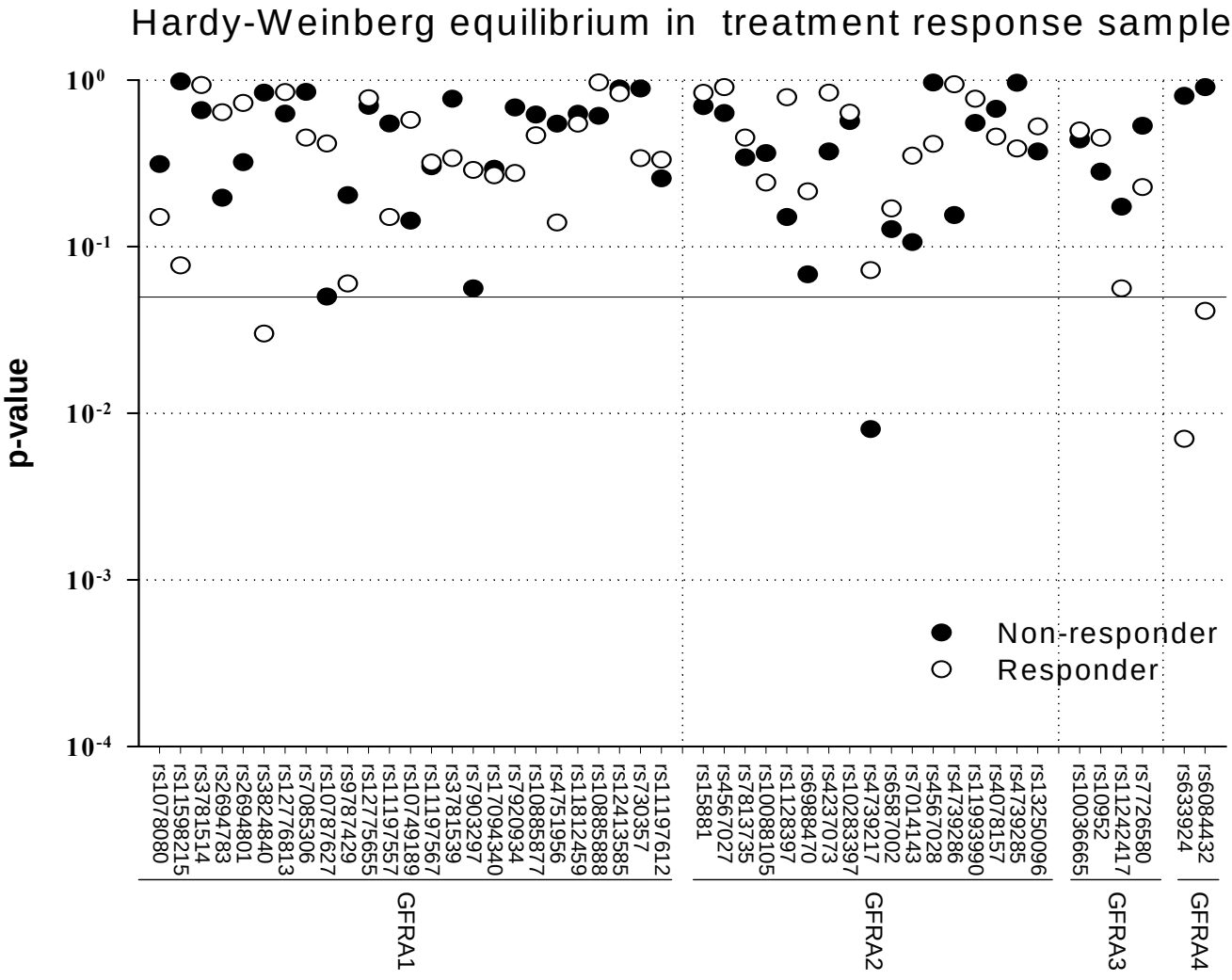

Hardy-Weinberg equilibrium in case-control sample
p-value
10^0
10^-1
10^-2
10^-3
10^-4
Control
Case
rs1078080
rs11598215
rs3781514
rs2694783
rs2694801
rs3824840
rs12776813
rs7085306
rs10787627
rs9787429
rs12775655
rs11197557
rs10749189
rs11197567
rs3781539
rs7903297
rs17094340
rs7920934
rs10885877
rs4751956
rs11812459
rs10885888
rs12413585
rs730357
rs11197612
rs15881
rs4567027
rs7813735
rs10088105
rs1128397
rs6988470
rs4237073
rs4739217
rs10283397
rs6587002
rs7014143
rs4567028
rs4739286
rs11993990
rs4078157
rs4739285
rs13250096
rs10036665
rs10952
rs11242417
rs726580
rs6084432
rs633924
GFRA1
GFRA2
GFRA3
GFRA4

Figure 2:


Hardy-Weinberg equilibrium in treatment response sample
p-value
10^0
10^-1
10^-2
10^-3
10^-4
Non-responder
Responder
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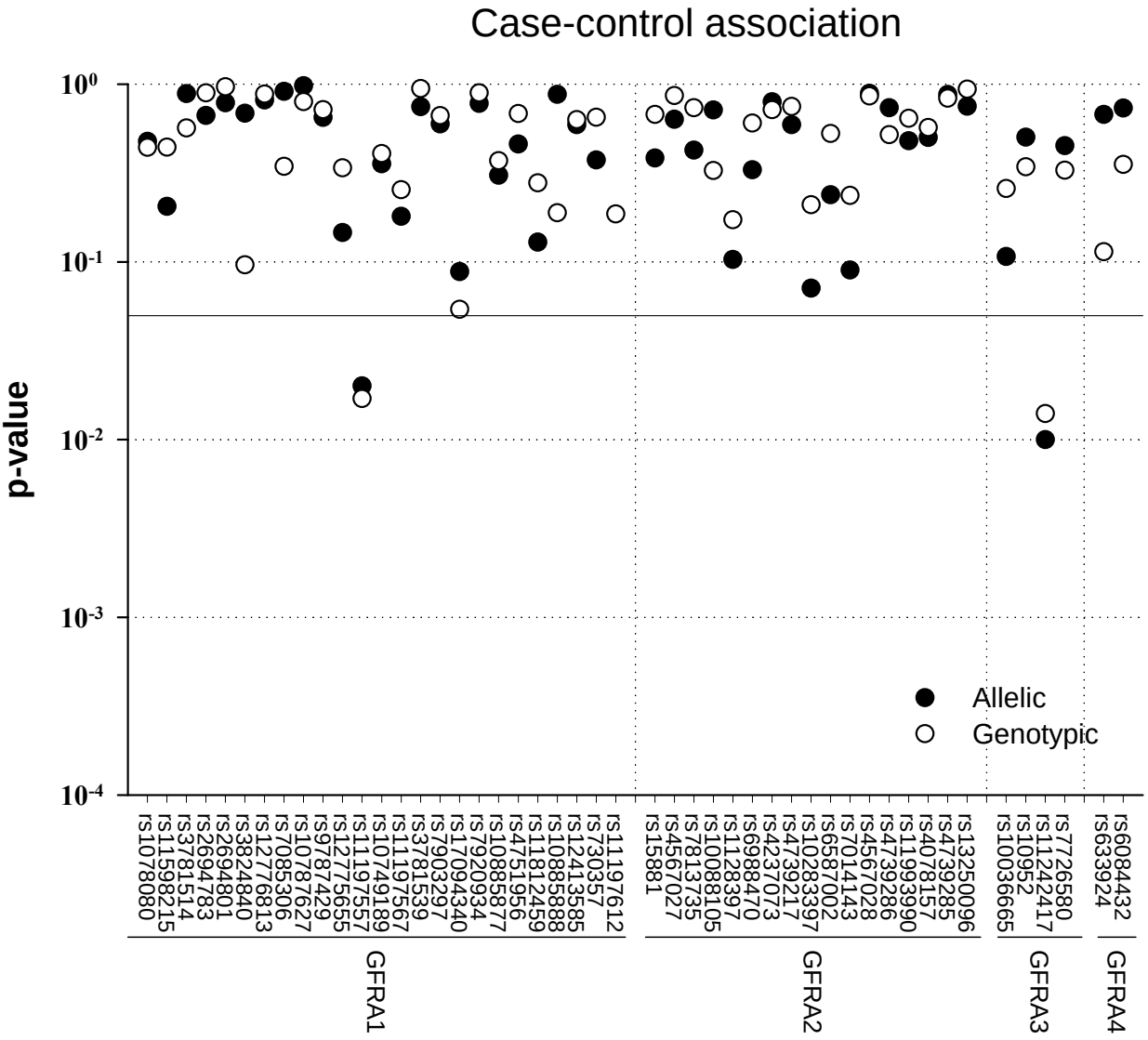
rs3781514
rs2694783
rs2694801
rs3824840
rs12776813
rs7085306
rs10787627
rs9787429
rs12775655
rs11197557
rs10749189
rs11197567
rs3781539
rs7903297
rs17094340
rs7920934
rs10885877
rs4751956
rs11812459
rs10885888
rs12413585
rs730357
rs11197612
GFRA1
rs15881
rs4567027
rs7813735
rs10088105
rs1128397
rs6988470
rs4237073
rs10283397
rs4739217
rs6587002
rs7014143
rs4567028
rs4739286
rs11993990
rs4078157
rs4739285
rs13250096
GFRA2
rs10036665
rs10952
rs11242417
rs726580
GFRA3
rs633924
rs6084432
GFRA4

Figure 3


Case-control association
p-value
10^0
10^-1
10^-2
10^-3
10^-4
Allelic
Genotypic
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rs2694801
rs3824840
rs12776813
rs7085306
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rs9787429
rs12755655
rs11197557
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rs17094340
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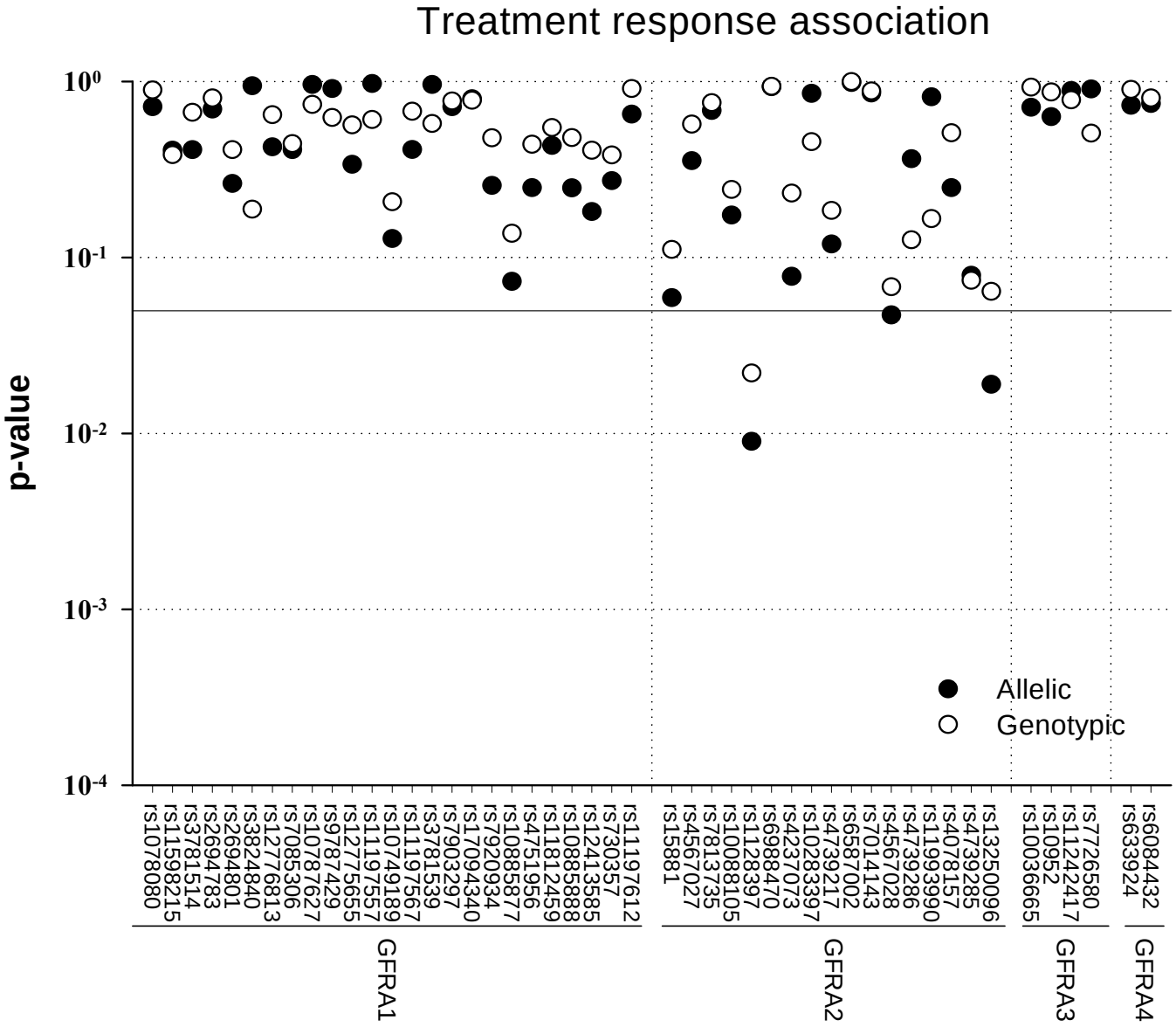
rs4567028
rs4739286
rs11993990
rs4078157
rs4739285
rs13250096
rs10036665
rs10952
rs11242417
rs7726580
rs633924
rs6084432
GFRA1
GFRA2
GFRA3
GFRA4

Figure 4


Treatment response association
p-value
10^0
10^-1
10^-2
10^-3
10^-4
Allelic
Genotypic
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rs11598215
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rs2694801
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rs12776813
rs7085306
rs10787627
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rs4567028
rs4739286
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rs4078157
rs4739285
rs13250096
rs10036665
rs10952
rs11242417
rs726580
rs6084432
rs633924
GFRA1
GFRA2
GFRA3
GFRA4

Figure S$

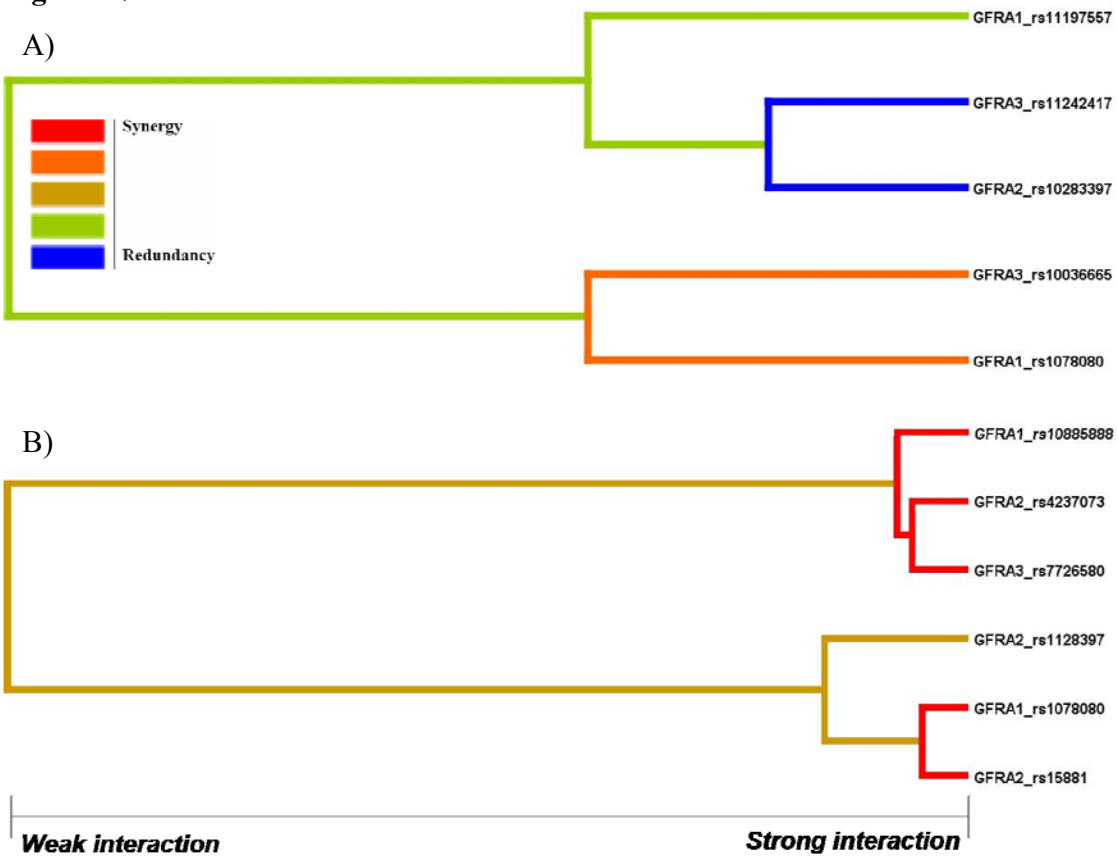
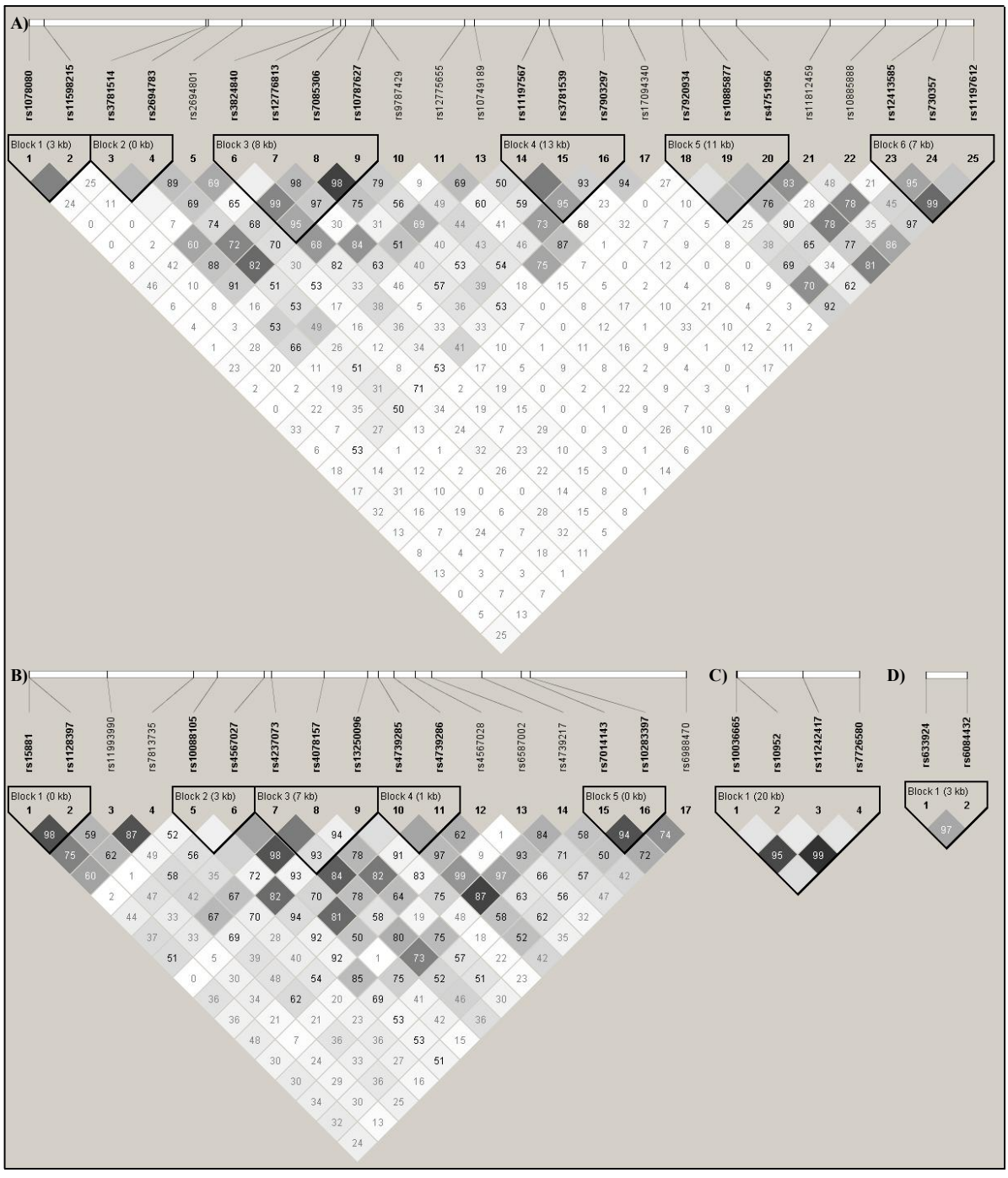

A)
Synergy
Redundancy
GFRA1_rs11197557
GFRA3_rs11242417
GFRA2_rs10283397
GFRA3_rs10036665
GFRA1_rs1078080
B)
GFRA1_rs10885888
GFRA2_rs4237073
GFRA3_rs7726580
GFRA2_rs1128397
GFRA1_rs1078080
GFRA2_rs15881
Weak interaction
Strong interaction

Figure S1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rs3781514
rs2694783
rs2694801
rs3824840
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rs7085306
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rs12775655
rs10749189
rs11197567
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rs7903297
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B)
rs15881
rs1128397
rs11993990
rs7813735
rs10088105
rs4567027
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rs10283397
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C)
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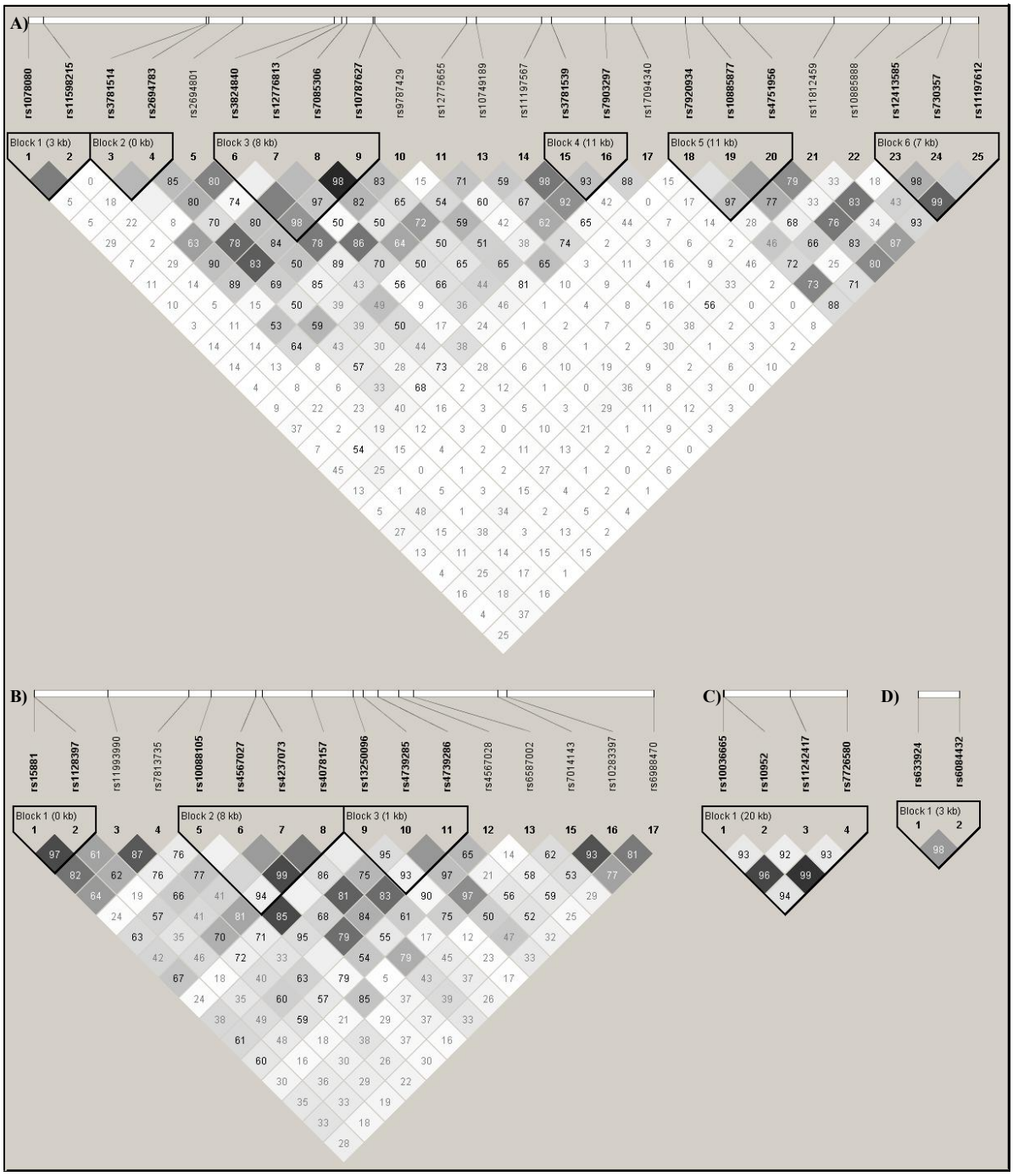
D)
rs633924
rs6084432
Block 1 (3 kb)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rs11598215
rs3781514
rs2694783
rs2694801
rs3824840
rs12776813
rs7085306
rs10787627
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rs10749189
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rs4567027
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rs4078157
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rs4567028
rs6587002
rs7014143
rs10283397
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Block 3 (1 kb)
C)
rs10036665
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rs7726580
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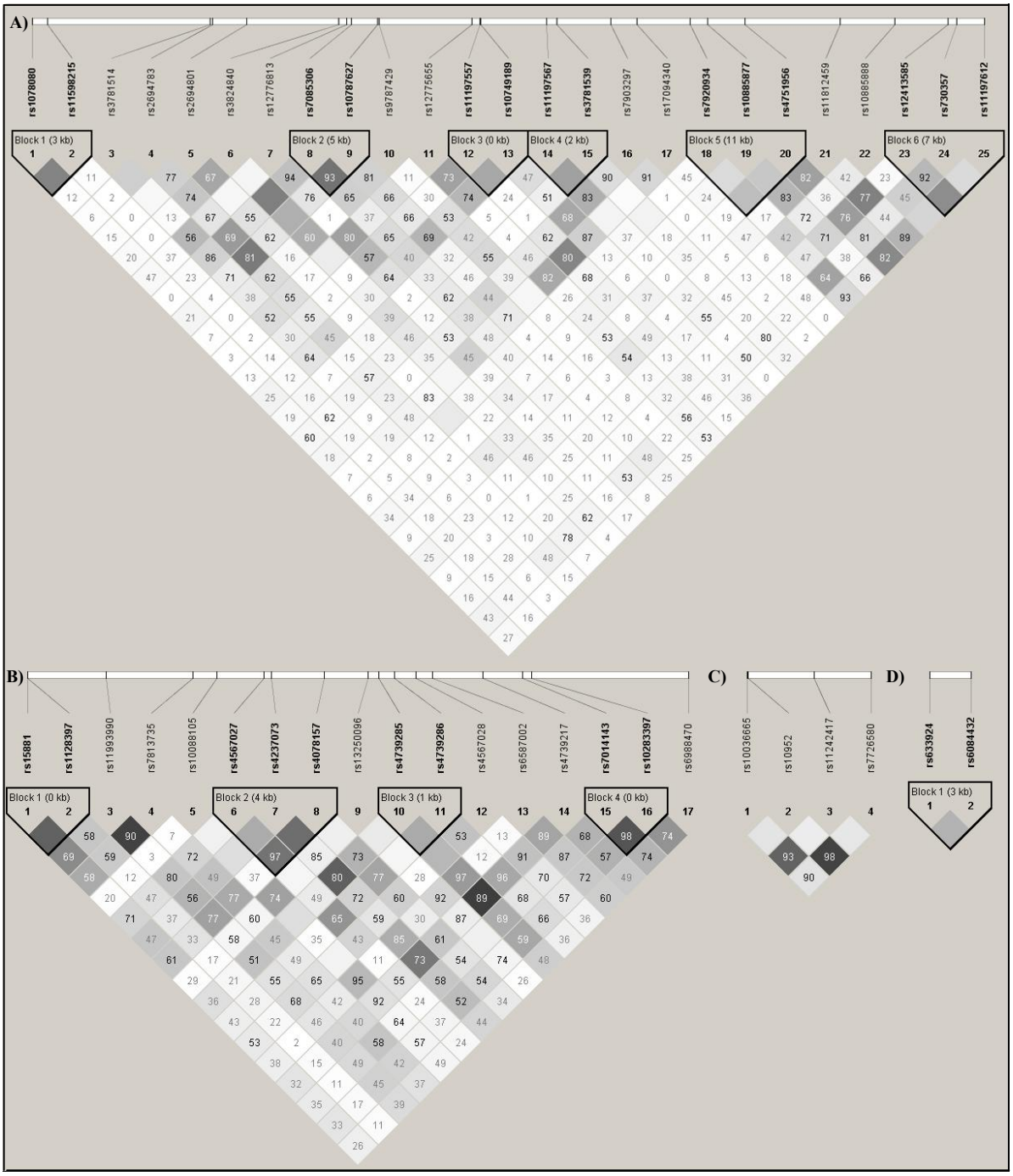
D)
rs633924
rs6084432
Block 1 (3 kb)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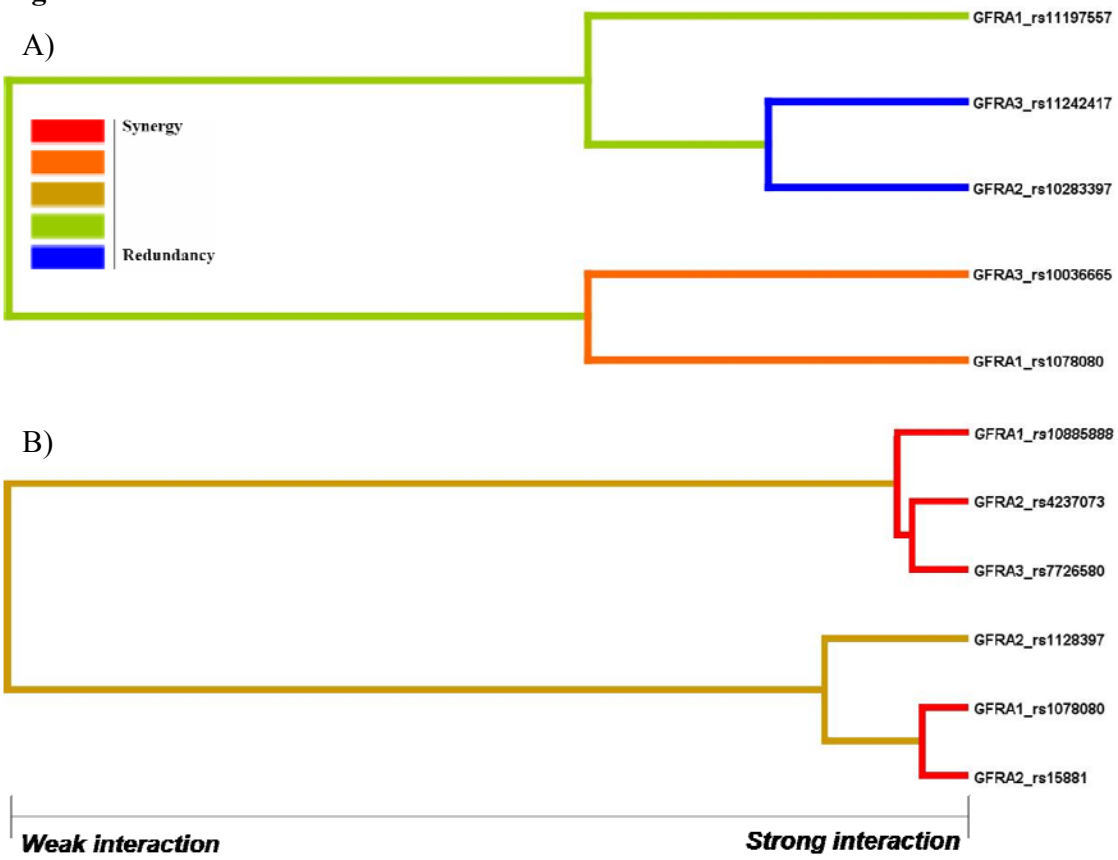
D)
rs633924
rs6084432
Block 1 (3 kb)

Figure S4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Synergy
Redundancy
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GFRA3_rs11242417
GFRA2_rs10283397
GFRA3_rs10036665
GFRA1_rs1078080
B)
GFRA1_rs10885888
GFRA2_rs4237073
GFRA3_rs7726580
GFRA2_rs1128397
GFRA1_rs1078080
GFRA2_rs15881
Weak interaction
Strong interaction

Legends

Table 1: Allelic and genotypic frequencies for the case-control association

Table 1: (continued)

Table 2: Family –based association test. S represents the test statistic for observed number of alleles, E represents the expected value of S under null hypothesis and Var(S) represents variance between the observed and expected transmission.

Table 3: Allelic and genotypic frequencies for the clozapine response association

Table 3:(continued)

Figure 1: Hardy-Weinberg equilibrium p-values for cases and controls. The solid line represents p = 0.05

Figure 2: Hardy-Weinberg equilibrium p-values for the clozapine non-responders and responders. The solid line represents p = 0.05

Figure 3: Allelic and genotypic p-values for the case-control association. The solid line represents p = 0.05

Figure 4: Allelic and genotypic p-values for the clozapine response association. The solid line represents p = 0.05

Figure S1: LD plot for the analyzed markers in GFRA1, GFRA2, GFRA3 and GFRA4 in case-control (A, B, C, D); respectively. Values presented are the D'.

Figure S2: LD plot for the analyzed markers in GFRA1, GFRA2, GFRA3 and GFRA4 in family sample (A, B, C, D) respectively. Values presented are the D'.

Figure S3: LD plot for the analyzed markers in GFRA1, GFRA2, GFRA3 and GFRA4 in response sample (A, B, C, D) respectively. Values presented are the D'.

Figure S4: Interaction dendrogram in case-control (A) and clozapine response (B) samples.

3.3 - Clinical involvement of oxidative stress genes polymorphisms in schizophrenia: influence on the severity of symptoms and response to clozapine treatment

Clinical involvement of oxidative stress genes polymorphisms in schizophrenia: influence on the severity of symptoms and response to clozapine treatment

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Abstract

Abnormal activities of critical antioxidant enzymes and other indices of lipid peroxidation in plasma and red blood cells have been detected in patients with schizophrenia. Other results have shown that oxidative stress may be modulated by clozapine. Based on that and some studies already found different clinical relations between reactive oxygen species and negative and positive symptoms, in the present study, it was studied the association between clinical response and the polymorphism in the GPX1 (Pro197Leu) and MNSOD (Ala16Val) gene in 216 clozapine-treated patients with schizophrenia. No association was found with these two functional polymorphisms and clozapine response change after six months, not even using a gene-gene interaction model. No correlations were found between positive/negative symptoms score and both polymorphisms. Our results present that GPX1 (Pro197Leu) and MNSOD (Ala16Val) polymorphisms seem do not play a central role in the clozapine response, although studies in larger and independent samples are necessary to confirm our findings.

Introduction

Schizophrenia is a devastating psychiatric disorder that affects around 1% of the population. It has been shown to be a complex multifactorial disease with both genetic and environmental influences. Antipsychotic drugs are the best means available for symptomatically treating individuals suffering from schizophrenia; however, there is a significant variability in clinical response to these psychotropic medications. Take, for example, clozapine, the prototype atypical antipsychotic, where only 30–60% of individuals resistant to typical antipsychotics may demonstrate a beneficial clinical response with respect to positive and negative symptomatology (Malhotra et al. 2004)

Human glutathione peroxidase (GPX1; OMIM#138320) is a selenium-dependent enzyme ubiquitously expressed and is found in cytoplasm and mitochondria which plays an important role in detoxification of free radicals (Ursini et al., 1985). GPX1 knockout mice show increased susceptibility to oxidative stress-inducing agents (paraquat and hydrogen peroxide) (de Haan et al., 1998). The human GPX1 gene has been located on

chromosome 3p21.3 (Kiss et al., 1997), and it is composed of two exons within a 1.42 Kb region (Ishida et al., 1987). A single nucleotide polymorphism (SNP) in the GPX1 gene has been reported at nucleotide 593, C to T substitution which causes a proline (Pro) to leucine (Leu) substitution at codon 197 (Pro197Leu) (Forsberg et al., 1999). The effect of the Pro197Leu polymorphism on the function of GPX1 enzyme is considerable. Although one study reported that erythrocyte GPX1 activity showed no significant difference between the genotypes (Forsberg et al., 2000), a more recent study using human transfected cells, which exclusively express either the Pro- or Leu-containing GPX1 allele, showed functional differences between the two alleles (Leu-containing allele was less responsive to the stimulation of GPX1 enzyme activity). (Hu and Diamond, 2003).

Superoxide dismutases (SOD) are the only enzymes that convert superoxide radicals to hydrogen peroxide. The genes encoding these enzymes are located in different chromosomes and in all of them polymorphisms have been described. Copper-zinc SOD (CuZnSOD, SOD1) is encoded on 21q22.1 (OMIM#147450), manganese SOD (MnSOD, SOD2) on 6q25.3 (OMIM#147460), and extracellular SOD (ECSOD, SOD3) on 4p16.3–q21 (OMIM#185490). MnSOD is synthesized in the cytoplasm as a precursor molecule containing a leader signal that is later removed during the transport of the molecule to the mitochondria (Weisiger and Fridovich., 1973ab, Shimoda-Matsubayashi et al., 1996). Ala16Val SNP is common (Val allele frequency approximately 48%) and it has been suggested that it may change the secondary structure and mitochondrial targeting of the protein (Wang et al., 2001).

Abnormal activities of critical antioxidant enzymes (Reddy et al. 1991; Vaiva et al. 1994; Altuntas et al. 2000) and other lipid peroxidation parameters (Mahadik et al. 1995; Kuloghi et al. 2002; Arvindakshan et al., 2003a) in plasma and red blood cells have been detected in patients with schizophrenia. Mahadik found increased lipid peroxidation products and altered defence system in both chronic and drug-naive first episode schizophrenia patients (Mahadik et al., 1996). Zhang performed analyses in some enzymes related with oxidative stress in subjects with schizophrenia including paranoid, disorganized and residual subtypes. They found that activities of SOD and GPX were decreased but levels of malondialdehyde were elevated in patients with a chronic form of schizophrenia as compared with normal controls. SOD and GPX activities were found to be significantly lower in paranoid and residual subtypes compared to both disorganized subtype and the control group. Malondialdehyde levels were significantly higher in all subtypes compared to control group (Zhang et al., 2006).

Although classic antipsychotic drugs such as haloperidol produce a marked reduction in positive symptoms of schizophrenia, they do not improve the negative symptoms such as apathy, confusion, and social withdrawal, nor do they alter the progressive deterioration in the mental abilities of the patient. Meanwhile, atypical antipsychotics have been shown to improve both positive and negative symptoms of schizophrenia, and seem to prevent further worsening of psychotic symptoms (Buckley, 1997; Blin, 1999). The mechanisms by which clozapine exerts its antipsychotic actions in schizophrenia likely involve the blockade of dopamine and serotonin receptors; however, the molecular mechanisms by which clozapine and the other atypical antipsychotics prevent symptom progression remain to be determined. Some results indicate that oxidative stress is integral to this disease and not the result of neuroleptic treatment although antipsychotic-induced oxidative stress results in rat or cell lines are not

conclusive (Polydoro et al., 2004; Reinke et al., 2004; Agostinho et al., 2007; Pillai et al., 2007; Streck et al., 2007).

Several clinical studies that analyzed oxidative parameters and antipsychotic drugs tried to relate these features with tardive dyskinesia. Just some studies had been published relating oxidative stress with clinical response or psychiatric scales scores during the treatment with antipsychotics. Arvindakshan reported reduction in brief psychiatric rating scale (BPRS) and positive and negative syndrome scale (PANSS) score after supplementation with antioxidants agents, such as omega-3 fatty acids, vitamin C, and vitamin E. It was shown that red blood cells SOD is increased in positive schizophrenia (Crow's type I), but not in Crow's type II. This finding means that patients with schizophrenia with positive symptoms are faced with increased oxidative stress indicating that maybe response of oxidative stress could be differently related with positive and negative symptoms (Arvindakshan et al., 2003b). Based on the that oxidative stress could be modulated by clozapine and some studies already found different clinical relations between ROS and negative and positive symptoms, in the present study, we studied the association between clinical response and the polymorphism in the GPX1 (Pro197Leu) and MNSOD (Ala16Val) gene in clozapine-treated patients with schizophrenia.

Methods

Clinical sample

Clinical data from 216 patients with DSM-III-R or DSM-IV diagnoses of schizophrenia, almost all of whom met criteria for treatment refractoriness or intolerance to typical antipsychotic therapy (Souza et al. 2008), were obtained at the following research clinics: Case Western Reserve University in Cleveland, OH (Meltzer, n=100); Hillside Hospital in Glen Oaks, NY (Lieberman, n=87); University of California at Irvine (Potkin, n=29). After informed consent was obtained, patients underwent a washout period of 2 to 4 weeks during which, unless clinically necessary, they received no medications before starting clozapine. Clozapine treatment was continued for a minimum of 6 months during which patients were evaluated prospectively. Clozapine blood levels were monitored through- out the course of treatment to ascertain compliance. Treatment response was evaluated as a % score change using the 18 item Brief Psychiatric Rating Scale (BPRS), a four item (conceptual disorganization, suspiciousness, hallucinations, unusual thought content) positive symptom subscale (BPOS) and a three item (emotional withdrawal, motor retardation, blunted affect) negative symptom subscale (BNEG) after 6 months of clozapine treatment from enrolment into the study (baseline) (with a negative value indicating an improvement in symptoms): % Score Change=(6 Months Score Baseline Score) / (Baseline Score). When treatment response was evaluated as a dichotomous variable in the whole sample at 6 months using criteria based on those of Kane et al. (1988): a reduction of ≥20% on the overall score of the BPRS from the baseline score taken at enrolment into the study.

Genetic analyses

Genomic DNA was extracted using the high salt method of Lahiri and Nurnberger (1991). GPX1 genotypes were assessed by the TaqMan allele specific assay method (Applied Biosystems, Foster City, CA) according to the manufacturer's protocols. The Pro197Leu polymorphism site was amplified by polymerase chain reaction (PCR) using the following primers: 5`-CATCGAAGCCCTGCTGTCT-3' (forward) and 5'-CACTGCAACTGCCAAGCA-3' (reverse). Genotyping was performed by 5'-exonuclease fluorescence assay. All genotypes were reported with the allelic discrimination program using the ABI software and confirmed by two experienced researchers. Samples which gave ambiguous calls were genotyped again. MnSOD genotypes were assessed by restriction fragment length polymorphism (RFLP) using the following primers 5'-AGCCCAGCCGTGCGTAGAC-3' and 5'-TACTTCTCCTCGGTGACG-3' and the PCR product was digested with BsaWI enzyme.

Statistical analyses

Individual SNP analyses of responder (control)/non-responder (case) data were performed using χ^2 tests. Individual SNP analyses of % score changes (continuous data) were performed using Analysis of Variance (ANOVA). The statistical program used was the Statistical Package for the Social Sciences, version 10.0.7. The nonparametric Multifactor Dimensionality Reduction (MDR) approach was selected for the analysis of gene–gene interaction (Moore et al., 2006).

Results

Demographic data

Demographic distribution of clinical sites is presented in Table 1. There were no differences observed between the sites in terms of gender ratio, mean age or response ratio. When compared Caucasians and African-American population were not found significant differences in terms of gender ratio, mean age or response ratio.

Genotype data

Significant deviation from Hardy–Weinberg equilibrium was observed for GPX1 polymorphism (non-responders p= 0.002 and responders p=0.029). No deviations were observed for MnSOD polymorphism (non-responders p= 0.606 and responders p=0.433). Responder/non-responder groups were compared for genotype and allele frequencies (see Table 2). In our population, no significant differences were observed for genotype or allele frequency comparisons between responders and non-responders for any of the studied SNPs. No significant association was found either in just the Caucasian or African-American population (data not shown).

Scale score data

BPRS, BPOS and BNEG basal and percentage change score distributions of genotype groups were compared against each other for each SNP (see Table 3).

Lieberman sample scale score data was not available so the results are relative of Meltzer and Potkin samples. No significant associations were observed in any of the scores. No associations were also found either in just the Caucasian or African-American population (data not shown).

Gene-gene interaction

After we did not find any association with single SNP and clozapine response we checked if together these two genes would be able to predispose clozapine response. MDR analyzes showed that when both genes are together there is a synergic effect (see Figure 1) but this did not reach significant association level (p=0.179).

Discussion

Over 50 years ago, Hoffer, Osmond and Smythies proposed that schizophrenia may be associated with free radical (i.e., reactive oxygen species, ROS) mediated pathology (Hoffer et al., 1954). Contemporary knowledge in neurochemistry increasingly emphasises the role of free radicals in the genesis of structural and functional changes of neuronal membrane that could be responsible for the beginning or aggravation of some diseases. The nervous system possess high potentials for the initiation of free radical reactions (large amount of unsaturated fatty acids, catecholamines and monoamines), which, relative to other tissues, can cause more damage in the brain and nervous system due to insufficient antioxidative protection and existing intensive aerobic metabolism accompanied with oxygen radical production.

Mahadik found increased lipid peroxidation products in both chronic and drug-naive first episode schizophrenia patients (Mahadik et al., 1996). The effect of oxidative modification of neuronal phospholipids, DNA, and proteins on their function (i.e.membrane transport, loss of mitochondrial energy production, gene expression and, therefore, receptor-mediated phospholipid-dependent signal transduction) may explain altered information processing in schizophrenia and changes in these oxidative process could be attributed to antipsychotic drugs. Although exact mechanism is not known, direct effect of drugs on lipid peroxidation or indirect effect through alteration in superoxide and hydroxy radical formation could not be ruled out. Atypical antipsychotic like clozapine seems to increase 5-hydroxyindol acetic acid (5-HIAA), which is excellent scavenger of hydroxyl and superoxide radicals (Blakely et al. 1984; Liu and Mori 1993). Some *in vitro* and *in vivo* studies in animals indicate that treatment with some atypical antipsychotics may be neuroprotective against oxidative cell injury by inducing antioxidant protection (Li et al., 1999; Parikh et al., 2003; Pillai et al., 2007). Our results could not show any association with GPX1 and MnSOD polymorphisms with clozapine response either isolated or after gene-gene interaction analyze.

BPRS score is significantly reduced after supplementation of vitamin C compared to placebo as substantiated by significant and negative correlation between BPRS score and plasma ascorbic acid levels (Dakhale et al., 2005). Recently, one study demonstrated reduction in BPRS and PANSS and increase in Henrich's quality of life scale score after supplementation with omega-3 fatty acids, vitamin C, and vitamin E (Arvindakshan et al. 2003b). Ascorbic acid is a water-soluble ketoacetone. It plays a crucial role in the

suppression of superoxide radicals by blocking catecholamine autooxidation (Cadet and Brannock, 1997) thereby inhibiting formation of potential toxic by-products such as 6-hydroxy dopamine (6-OHDA), semiquinone, hydrogen peroxide, and hydroxyl radical, eventually leading to neuronal damage in the brain and development of defect symptoms (Cadet and Lohr, 1987). This could be one of the reasons for reduction in BPRS score after vitamin C supplementation. We did not report any significant association connecting BPRS score and percentage change after 6 months of clozapine treatment and GPX1/ MnSOD.

It has been shown that patients with schizophrenia with positive symptoms are faced with increased oxidative stress (Pavlovic et al., 2002), however in our sample we could not find any association with these two functional polymorphisms in key oxidative stress enzymes and the positive symptoms or with its change after six months. Sirota related a positive correlation between superoxide generation and negative symptoms in patients with schizophrenia supporting the hypothesis that superoxide anion may participate in the pathogenesis of schizophrenia, as an excess of free radicals could contribute to the deterioration phase of the disease (Sirota et al., 2003). We also did not show correlation with the negative symptoms and these two oxidative stress-related genes. In summary, although some studies reported associations among oxidative stress and schizophrenia clinical features/ antipsychotic activity, in this present study we did not show any correlation linking GPX1 and MnSOD gene polymorphisms and these features. Further studies with a larger sample are necessary to confirm these negative findings.

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Figures and tables

Sample (n)	Male/female [*n* (%)]	Age (mean ±SD)	Responder/non-responder [*n* (%)]	Caucasian/ African-American [*n*(%)]
Meltzer (100)	72/28 (72/28)	33±9	49/51 (49/51)	74/26 (74/26)
Lieberman (87)	62/25 (71/29)	35±8	52/35 (60/40)	72/15 (83/17)
Potkin (29)	22/07 (76/23)	35±7	11/18 (38/62)	25/04 (86/14)
Caucasian (171)	124/47 (73/27)	35±18	86/85 (50/50)	-
African-American (45)	31/14 (69/31)	34±10	26/19 (58/42)	-

Table 1: Demographic characteristics and percentage response in each population.

Marker	Allele frequency						Genotype frequency						Association			
													Allele		Genotype	
	Non-responder			Responder			Non-responder			Responder			Chi-square	p-value	Chi-square	p-value
		n	%		n	%		n	%		n	%				
GPX1	1	47	27.6	1	48	25.0	11	1	1.2	11	2	2.1	0.33	0.567	1.03	0.595
	2	123	72.4	2	144	75.0	12	45	52.9	12	44	45.8				
							22	39	45.9	22	50	52.1				
MnSOD	1	84	44.7	1	99	48.5	11	20	21.3	11	26	25.5	0.58	0.445	0.57	0.751
	2	104	55.3	2	105	51.5	12	44	46.8	12	47	46.1				
							22	30	31.9	22	29	28.4				

Table 2: Individual SNP non-responder/responder analyses: frequencies and significance levels

Figure 1: Interaction dendogram

Gene	Parameter	Time point	Geno type	n	Mean	SD	95%CI		F	p-value
							Lower	Upper		
GPX1	BPRS	basal	11	3	44.67	8.38	23.83	65.50	0.538	0.586
			12	51	35.94	14.82	31.77	40.11		
			22	52	35.67	14.66	31.59	39.76		
		% 6 months	11	3	-35.90	47.44	-153.7	81.96	0.379	0.686
			12	51	-19.67	29.01	-27.83	-11.51		
			22	51	-20.99	33.03	-30.28	-11.70		
	BPOS	basal	11	3	13.67	5.13	0.92	26.41	0.405	0.668
			12	51	13.29	6.35	11.51	15.08		
			22	52	12.25	5.94	10.60	13.90		
		% 6 months	11	3	-35.20	56.18	-174.7	104.38	0.206	0.815
			12	47	-20.20	56.53	-36.80	-3.60		
			22	50	-16.09	54.23	-31.51	-0.68		
	BNEG	basal	11	3	8.00	5.00	-4.42	20.42	0.479	0.621
			12	51	6.10	3.71	5.05	7.14		
			22	52	6.79	5.11	5.36	8.21		
		% 6 months	11	3	14.43	84.61	-195.7	224.63	0.734	0.483
			12	48	11.48	111.3	-20.86	43.82		
			22	50	-9.34	54.85	-24.93	6.25		
MnSOD	BPRS	basal	11	28	39.61	18.26	32.53	46.69	0.406	0.668
			12	48	36.79	13.86	32.77	40.82		
			22	36	36.33	15.24	31.17	41.49		
		% 6 months	11	27	-23.10	30.12	-35.01	-11.18	0.241	0.786
			12	48	-20.93	29.70	-29.55	-12.30		
			22	36	-17.81	32.15	-28.68	-6.93		
	BPOS	basal	11	28	13.25	6.76	10.63	15.87	0.007	0.993
			12	49	13.14	6.31	11.33	14.96		
			22	36	13.06	6.09	10.99	15.12		
		% 6 months	11	27	-19.10	45.25	-37.00	-1.20	0.180	0.835
			12	47	-14.93	69.78	-35.42	5.56		
			22	34	-22.50	41.86	-37.11	-7.90		
	BNEG	basal	11	28	7.25	5.70	5.04	9.46	0.183	0.833
			12	49	6.65	4.87	5.25	8.05		
			22	36	7.19	4.36	5.72	8.67		
		% 6 months	11	27	-9.60	60.15	-33.40	14.20	0.519	0.597
			12	46	11.82	109.7	-20.76	44.41		
			22	35	1.99	69.29	-21.81	25.80		

Table 3: Basal and after 6 months percentage change scores

3.4 - Genetic association study of NALCN polymorphisms with schizophrenia and antipsychotic treatment

Genetic association study of NALCN polymorphisms with schizophrenia and antipsychotic treatment

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Running title: *NALCN* schizophrenia, treatment response and side-effects

Abstract

NALCN (sodium leak channel, non-selective) is a gene located on chromosome 13q in a suggested linkage region for schizophrenia. Mouse NALCN mediates some background sodium leak in hippocampal neurons and plays a role in neuronal excitability. Abnormalities in hippocampal activity and neuronal excitability have been implicated in schizophrenia. In this study we analyzed 26 *NALCN* polymorphisms and examined association with four phenotypes: diagnosis of schizophrenia (case-control and family-based analysis), clozapine response, clozapine-induced weight gain and antipsychotic-induced tardive dyskinesia (TD) in schizophrenia patients. We compared allele; genotype and haplotype frequencies in 219 matched case-control subjects, 85 small nuclear families, 150 schizophrenia patients taking clozapine for 6 months, 67 patients with weight gain accessed after 6 weeks of clozapine treatment and 210 patients with antipsychotic-induced TD. In case-control samples we found significant associations with rs9518320 and rs9518331 and haplotypes composed of rs7317836, rs9518320, rs9518331, rs2584531 and rs3916906. We did not find any significantly altered transmission in the schizophrenia family sample. Our results showed rs2152324 to be associated with clozapine response. A haplotype formed by rs10508059-rs7328287-rs496238 showed association with clozapine-induced weight gain. Five individual SNPs (rs9513851, rs9518307, rs9518349, rs10508059 and rs7328287) and haplotypes composed of rs9513851-rs9518307 and rs7328287-rs496238) showed significant associations with TD. Our results suggest that the *NALCN* may affect susceptibility to schizophrenia, antipsychotic response and side-effects.

Keywords: schizophrenia, clozapine response, *NALCN,* ion channels, genetic association, family-based association test.

Introduction

Schizophrenia affects about 1.0% of the population worldwide, with devastating consequences for both patients and their families and is the seventh most costly medical illness (Freedman, 2003). The hallucinations, delusions, thought disorder, and cognitive deficits associated with schizophrenia impact profoundly on the perception, emotion, and judgment of patients. Current treatments are only partially successful, and therefore the development of novel treatments based on an understanding of the etiology and pathogenesis of schizophrenia is imperative. Until recently, progress in schizophrenia research has been limited by a number of factors, including disease heterogeneity and the lack of clear pathological lesions. Evidence increasingly suggests that schizophrenia is a disorder of brain development and plasticity. Genetic studies have recently begun to identified strong candidate risk genes for schizophrenia, and neurobiological studies of the normal and variant forms of these genes are advancing (Owen et al, 2005; Craddock et al, 2005; Chen et al, 2006; Riley and Kendler, 2006; Ross et al, 2006).

Linkage and association studies have implicated several loci in the genome that likely harbor genes conferring risk for schizophrenia. The interpretation of genetic linkage results is controversial and some degree of subjectivity enters into the determination of which regions of the genome should be considered to have truly significant evidence for linkage to schizophrenia. Two meta-analyses have summarized these findings (Badner and Gershon, 2002; Lewis, *et al* 2003). Badner and Gershon (2002) suggested the existence of susceptibility genes on chromosomes 8p, 13q and 22q, however 13q was not supported by Lewis et al (2003). Among other genes, the 13q region contains *G72* (or *DAOA* at 13q33.2). Several individual replication studies and a meta-analysis have supported the association of *G72* with schizophrenia though, as with other loci, the associated alleles and haplotypes are not identical across studies and some polymorphic variants are located outside of the gene (Chumakov et al, 2002; Detera-Wadleigh and McMahon, 2006).

An adjacent region (13q33.1) also contains other genes that have been associated with neuropsychiatric diseases, such as fibroblast growth factor 14 (*FGF14*) (van Swieten et al, 2003; Dalski et al, 2005) and tripeptidyl peptidase II (*TPPII*). (Radu et al, 2006). In this region, 13q33.1, 4.1Mb upstream of *G72* is located *NALCN* (also known as *VGCNL1)*. NALCN is a highly conserved protein in mammals (99% identity between human and rat). Close homologues are also found in invertebrates. For example, *D. melanogaster* has a single homologous gene named α1U (for unusual α1 subunit, 57% identity with human *NALCN*) (Littleton and Ganetzky, 2000). Two homologs also exist in *C. elegans* namely *nca-1* and *nca-2*; both with 48% identity to human *NALCN* (Humphrey et al, 2007). Both *nca* and *na* proteins are expressed specifically in the nervous system.

NALCN family proteins display high homology to the alpha-subunit of voltage-gated cation channels. In *Drosophila,* hypomorphic alleles of this family protein, na, that result in a reduced protein expression are viable and fertile, but have altered circadian rhythms (Lear et al, 2005; Nash et al, 2002). The *NALCN* mutant flies also have altered sensitivity to volatile anesthetics such as halothane (Krishnan and Nash, 1990; Mir et al., 1997). In *C. elegans*, the *nca* loss of function mutant is also viable and fertile. It has also

been associated with altered sensitivity to halothane (Humphrey et al,2007), as well as altered locomotion patterns and synaptic functions (Jospin et al, 2007; Yeh et al., 2008).

Originally cloned in 1999 from the rat brain, *NALCN* is expressed in many brain regions (Lu et al, 2007) of vertebrates. *NALCN* encodes a voltage-independent, nonselective, non-inactivating cation channel permeable to sodium, potassium and calcium when exogenously expressed in HEK293 cells (Lu et al, 2007). Deletion of *NALCN* in mice results in a severely disrupted respiratory rhythm characterized by periods of apnea and mutant pups die within 24 hours of birth. *In vivo*, the NALCN channel appears to be the main source of the background sodium leak in the hippocampal neurons at rest and is important for neuronal excitability (Lu et al, 2007). Both hippocampal activity and neuronal excitability are processes strongly altered in schizophrenia (Saugstad, 1994; Oxley et al, 2004; Eichhammer et al, 2004; Goldman and Mitchell, 2004; Boyer et al, 2007). The effect of this ion channel on the daily rhythms of the fly and mouse are also especially relevant because of the circadian rhythm disturbances observed in schizophrenia and bipolar disorder (Christian et al. 2002). Because the function of *NALCN* is consistent with manifest some schizophrenia symptoms, and its location is within a suggestive chromosomal linkage region for schizophrenia, we hypothesized that *NALCN* may show a genetic association with schizophrenia, or the patient response to schizophrenia-treatment. To test this hypothesis, we performed an association study using both matched case-control and family samples. We also evaluated whether *NALCN* SNPs are associated with the alternate phenotypes clozapine response as well as two important side effects: clozapine-induced weight gain and antipsychotic-induced tardive dyskinesia (TD).

Methods

Clinical sample

All recruitment and clinical assessments were conducted with written informed consent and approval of our institutional ethics review board. Clinical data and DNA samples were obtained from the probands of 85 small nuclear families, as well as 219 patients with a DSM-III-R or DSM-IV diagnosis of schizophrenia. Healthy controls (N=219) were matched for age (±5 years), ethnicity and gender (146 male and 73 female cases and controls: mean age 36±8). The Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I) was administered by trained research assistants to each patient and diagnosis was supplemented by a review of medical records. The diagnosis was established via consensus procedures by two of the investigators. Controls were screened for current or past history of major psychiatric disorders or substance misuse, and excluded if either was detected.

For the clozapine response sample, clinical data from 140 patients with a DSM-III-R or DSM-IV diagnosis of schizophrenia, almost all treatment refractory or intolerant of typical antipsychotic therapy (Kane, *et al* 1988), were collected at Case Western Reserve University in Cleveland, OH (n=90) (63 males and 27 females, mean age 36±8) and Hillside Hospital in Glen Oaks, NY (n=50) (33 males and 17 females, mean age 35±8). After informed consent was obtained, recruited patients underwent a washout period of 2 to 4 weeks during which, unless clinically necessary, they received no

medications before starting clozapine. Clozapine treatment was continued for a minimum of 6 months during which patients' response was evaluated prospectively. Clozapine blood levels were monitored during the course of treatment to ascertain compliance. Treatment response was evaluated as the percentage score change on the 18-item Brief Psychiatric Rating Scale (BPRS). Treatment response was expressed as a dichotomous variable in the whole sample at 6 months using criteria based on those of Kane and coinvestigators (Kane, *et al* 1988): a reduction of ≥20% on the overall score of the BPRS from the baseline score at enrolment. There were no differences observed between the sites in terms of gender ratio, mean age, mean age of onset or response ratio. The Caucasian and African-American groups were not significantly different in terms of gender ratio, mean age, mean age of onset or response ratio (data not shown) (Hwang, *et al,* 2005).

For the clozapine-induced weight gain sample (n=67) were recruited from Case Western Reserve (38 males and 18 females, mean age 35±8) and from Hillside Hospital (4 males and 7 females, mean age 33±6). Weight gain at 6 weeks was expressed as a dichotomous variable using as criteria a weight increase ≥ 7% from baseline at enrolment. The FDA has established this threshold as producing a clinically meaningful and significant metabolic outcome.

For the TD samples, 210 subjects were recruited from four clinical sites: the Centre for Addiction and Mental Health in Toronto, ON (n=109; 71 males and 38 females, mean age 42±10); Case Western Reserve (n=60; 44 males and 16 females, mean age 33±10) and the Hillside Hospital (n=41; 25 males and 16 females, mean age 35±7). Regarding ethnicity, 16 subjects from Case Western and 38 from the Centre for Addiction and Mental Health were African-American; all other subjects were Caucasian. Subjects were selected with the same criteria used for the case-control sample. All patients had at least 1 year of cumulative treatment with typical antipsychotics. For the Case Western Reserve and Hillside Hospital samples, the presence or absence of TD was evaluated before any atypical antipsychotic administration. Patients in the Toronto TD sample were on various typical and atypical antipsychotics when TD was evaluated. TD was assessed using AIMS or the modified Hillside Simpson Dyskinesia Scale (HSDS) for patients recruited from the Hillside Hospital (Basile et al., 1999). The seven body area items and the overall global score of HSDS match those of AIMS; thus, TD assessment could be compared across all sites.

Genetic analyses

Genomic DNA was extracted using the high salt method as previously described (Lahiri and Nurnberger, 1991). We analyzed 25 SNPs in *NALCN* (rs1289556, rs9554752, rs17677552, rs686141, rs12867417, rs658213, rs614728, rs9513851, rs9518307, rs12584031, rs1452112, rs7317836, rs9518320, rs9518331, rs2584531, rs12869164, rs3916906, rs9518349, rs10508059, rs7328287, rs496238, rs7318529, rs9554772, rs17486808, rs17584161) and 1 SNP before promoter region (rs2152324). Genotyping was performed by GoldenGate assay (Illumina, San Diego, CA, USA) at The Centre for Applied Genomics (TCAG), Hospital for Sick Children in Toronto, ON, Canada.

Statistical analyses

Individual SNP analyses of case (schizophrenia patients; clozapine responders; weight gain greater than 7%; TD) and control (healthy controls; clozapine non-responders; absence of weight gain greater than 7%; no TD) data and Hardy–Weinberg equilibrium assessment were performed using χ^2 tests. The statistical program used was the Statistical Package for the Social Sciences, version 10.0.7 (SPSS 2000) for genotypic association and Haploview 4.0 (Barrett, 2005) for allelic association. We applied the family-based association test (FBAT, version 1.0, Laird, *et al* 2000) under the assumption of an additive model, and PEDSTATS (Wigginton and Abecasis, 2005) for Hardy–Weinberg equilibrium in the family data. Linkage disequilibrium (LD) was assessed using Haploview, version 4.0. Haplotype analyses were performed using UNPHASED 3.0.10 (Dudbridge, 2003), Haploview version 4.0 and FBAT.

Results

Linkage disequilibrium analysis

Pairwise LD between the SNPs is presented for each gene (Figure S1). In this study, we defined a haplotype block as a region over which less than 5% of pairwise comparisons among informative SNPs showed strong evidence of historical recombination (upper confidence bound on D′ less than 0.9; Gabriel, *et al* 2002). Based on this definition we found 5 LD blocks in the case-control sample, 7 in the family sample, 4 in the clozapine response sample, 3 in the clozapine-induced weight gain sample and 7 in the TD sample (figure S1).

Genotype data

Case-control sample: Significant deviation from Hardy–Weinberg equilibrium was observed for rs7318529 (p = 0.001) in control samples (Figure 1A). The cases and controls were compared for genotype and allele frequencies across the markers (Table 1 and Figure 1B). Significant associations were observed for: rs9518320 (allele p = 0.005, X^2 = 7.67; genotype p = 0.022, X^2 = 7.64) and rs9518331 (allele p = 0.006, X^2 = 7.42; genotype p = 0.029, X^2 = 7.10).

Family sample: Three SNPs showed significant deviation from Hardy–Weinberg equilibrium: rs9518320 (p = 0.023), rs9518331 (p = 0.022) and rs3916906 (p = 0.032), analyzing 80 unrelated individuals (Figure 1A). No significant associations were observed (Table 1 and Figure 1B).

Clozapine response sample: Significant deviations from Hardy–Weinberg equilibrium were observed for rs1758416 (p = 0.032) and rs2152324 (p = 0.029) in the non-responder group. The following markers deviated significantly from Hardy-Weinberg equilibrium in the responder group: rs1289556 (p = 0.010), rs9513851 (p = 0.043); rs9518307 (p = 0.043) and rs496238 (p = 0.044) (Figure 1A). Responder/non-responder groups were compared for genotype and allele frequencies across the markers (Table 1 and Figure 1B) and rs2152324 was associated with treatment response (allele p = 0.030, X^2 = 6.98; genotype p = 0.061, X^2 = 3.49).

Clozapine-induced weight gain sample: Significant deviations from Hardy–Weinberg equilibrium were observed for rs1050805 (p = 0.009) and rs2152324 (p = 0.023) in the patients with weight gain (Figure 1A). Patients with and without weight gain were compared for genotype and allele frequencies across the markers (see Table 1 and Figure 4) and no significant association was observed (Table 1 and Figure 1B).

Tardive dyskinesia sample: Two SNPs deviated significantly from Hardy-Weinberg equilibrium in patients without TD (rs9513581, p = 0.001 and rs10508059, p = 0.003), and one in patients with TD (rs9518307, p = 0.0006) (Figure 1A). Comparisons of allele and genotype frequencies revealed significant associations with five SNPs: rs9513851 (allele p = 0.008, X^2 = 9.75; genotype p = 0.031, X^2 = 4.60), rs9518307 (allele p = 0.053, X^2 = 5.87; genotype p = 0.049, X^2 = 3.86), rs9518349 (allele p = 0.042, X^2 = 6.33; genotype p = 0.308, X^2 = 1.03), rs10508059 (allele p = 0.032, X^2 = 6.86; genotype p = 0.097, X^2 = 2.74) and rs7328287 (allele p = 0.079, X^2 = 5.08; genotype p = 0.030, X^2 = 4.67) (Table 1 and Figure 1B).

Haplotype analysis

Case-control sample: Cases and controls were compared for haplotype frequencies. We did not find associations when considering haplotypes in the same LD block. We then performed three-marker sliding-window haplotype analysis across the whole gene in order to better characterize regions associated with our phenotypes. The following haplotypes showed association rs9518307-rs12584031-rs1452112 (global p = 0.015, X^2 = 13.98); rs7317836-rs9518320-rs9518331 (global p = 0.022, X^2 = 14.69; C-A-C control frequency = 0.439, case frequency = 0.371, p = 0.027, X^2 = 4.85; C-G-C control frequency = 0.234, case frequency = 0.320, p = 0.005, X^2 = 7.78) ; rs9518320-rs9518331- rs2584531 (global p = 0.047, X^2 = 14.21) and rs9518331-rs2584531-rs3916906 (global p = 0.045, X^2 = 12.87; A-G-A control frequency = 0.239, case frequency = 0.321, p = 0.008, X^2 = 6.82). The region including SNPs rs7317836, rs9518320, rs9518331, rs2584531 and rs3916906 showed significant associations for all three-marker sliding windows, and so we performed haplotype analysis with all these markers. The C-G-A-G-A haplotype showed significant association with schizophrenia (control frequency = 0.216, case frequency = 0.300, p = 0.004, X^2 = 8.01).

Family sample: There were no associations with haplotypes in the same LD block or three-maker sliding windows in the family-based sample.

Clozapine response sample: Clozapine responders and non-responders were compared for haplotype frequencies. No haplotypes were associated with clozapine response, either in the same LD block or in three-marker sliding windows.

Clozapine-induced weight gain sample: Subjects were compared for haplotype frequencies relative to weight gain (<7% or ≥ 7%), and no significant associations were detected with markers within the same LD block. Three-marker sliding window analysis showed one significant association: rs10508059-rs7328287-rs496238 (global p = 0.034, X^2 = 10.40; A-A-A control frequency = 0.163, case frequency = 0.030, p = 0.042, X^2 = 4.11).

TD sample: Subjects were compared for haplotype frequencies relative to the presence or absence of TD. Considering haplotypes in the same LD block, within block 3 (rs9513851 and rs9518307) we found association between haplotype C-A (TD absent

frequency = 0.922, TD present frequency = 0.969, p = 0.049, X^2 = 3.86; after 1,000 permutations p = 0.589) and A-G (TD absent frequency = 0.074., TD present frequency =, p = 0.025, X^2 = 4.60; after 1,000 permutations p = 0.430). Inside block 6 (rs7328287 and rs496238), the G-G haplotype showed association (TD absent frequency = 0.535, TD present frequency = 0.642, p = 0.030, X^2 = 4.67; after 1,000 permutations p = 0.409). No associations were found in the three marker sliding window analysis.

Discussion

This exploratory study examined the association of 26 SNPs in *NALCN* and four phenotypes: diagnosis of schizophrenia (using case-control and family-based analysis), clozapine response, clozapine-induced weight gain and antipsychotic-induced TD in schizophrenia patients. In case-control samples we found significant associations with rs9518320 and rs9518331 Furthermore, a 76.7Kb region containing rs7317836, rs9518320, rs9518331, rs2584531 and rs3916906 showed significant associations for all three-marker sliding window haplotypes. This region contains 186 SNPs in the CEPH HapMap population (Utah Residents with Northern and Western European Ancestry). Further analyses in this region are required to strengthen this association hypothesis. We did not find significantly altered transmission patterns with schizophrenia in our family sample.

Variation in individual clinical response to psychotropic drug treatment remains a critical problem in the management of serious mental illness (Basile, *et al* 2002; Malhotra, *et al* 2004). We examined if *NALCN* might play a role in treatment response and its side effects. Our results showed one SNP (rs2152324) with nominal association with clozapine response. One haplotype (rs10508059-rs7328287-rs496238 A-A-A) showed association with clozapine-induced weight gain. Regarding TD, five individual SNPs (rs9513851, rs9518307, rs9518349, rs10508059 and rs7328287) and three haplotypes (two with rs9513851-rs9518307 and one with rs7328287-rs496238) showed significant associations.

Corrections for multiple testing have been a controversial issue (Aickin, 1999; Bender and Lange, 1999; Perneger, 1998) and considering the exploratory nature of this study, without prespecified hypotheses for most of our SNPs, we assume no clear structure in the multiple tests. Therefore, our statistically significant results should properly be regarded as "exploratory", with confirmatory studies needed (Bender and Lange, 1999). Bonferroni correction for multiple testing on individual SNP associations renders all the associations non-significant (Bonferroni corrected $p<0.0019$). Nyholt (2004) correction was not performed because the gene size and low LD level observed among analyzed markers.

There were SNPs that deviated from Hardy-Weinberg equilibrium in the clozaopine response and antipsychotic-induced TD samples, as well as one SNP (rs7318529 p = 0.001) in healthy controls. We applied X^2 to test for genetic association in these SNPs, but the for low genotype counts the Fisher exact test, which does not rely on the X^2 null distribution approximation, is more appropriate (Guo and Thompson, 1992; Wigginton et al. 2005). With the Fisher exact test to evaluate Hardy–Weinberg equilibrium, we found a number of significant associations (rs1289556 in non-responders p=0.011; rs9513851 in non-responders p=0.151 and TD absent p = 0.037; rs9518307 in

non-responders p = 0.151 and TD present p = 0.061; rs10808059 in weight gain absent p = 0.019 and TD absent p = 0.007; rs496238 in responders p = 0.043; rs7318529 in healthy controls p = 0.011; rs17584161 in non-responders p =0.032 and weight gain absent p = 0.029; rs2152324 in non-responders p = 0.192). Assuming a significance threshold of $p<0.01$, one SNP remains deviated in the TD control sample (rs10508059). Trikalinos et al (2006) concluded that Hardy-Weinberg equilibrium should be routinely and transparently assessed in gene-disease association studies. Furthermore, discrepant results in these analyses do not necessarily mean that the observed association should be dismissed, but indicate the need for more evidence and validation. It is possible that deviations from HWE reflect disease associations (Nielsen, *et al* 1998; Wittke-Thompson, *et al* 2005; Balding, 2006).

NALCN is a 361.1Kbp gene with 44 exons located at chromosome 13q in a suggested linkage region for schizophrenia (Chumakov et al, 2002; Badner and Gershon, 2002; Christian et al, 2002). It has been show that *NALCN* mRNA is expressed in the cerebral cortex and hippocampus in all neurons and layers, and in all neurons of the spinal cord (dorsal and ventral horns). *NALCN* mRNA expression was not detected in liver, muscle, lung, kidney, or testis (Lee et al, 1999; Lu et al, 2007). *NALCN* mutant mouse neonates do not display gross abnormalities in embryonic development, righting responses, spontaneous limb movement, and toe/tail pinch responses, but do not survive beyond 24 hours after birth. Thus, NALCN is one of the few members of the family of four homologous repeat (domains I–IV), six transmembrane segment (S1–S6) (4x6TM) ion channels that are required for neonatal survival. Unlike any of the other 20 members in the 4x6TM channel family, NALCN forms a voltage-independent and non-inactivating cation channel (Lu et al, 2007).

NALCNs functions in establishing levels of neuronal excitability and thus controlling firing rates (Lu et al, 2007). Proteins that affect patterns of neuronal firing might play a part in the pathogenesis of schizophrenia (Miller et al, 2001; Mansvelder et al, 2006), and other examples include the human calcium-activated potassium channel (*SKCα3* also known as *KCNN3* and *SK3*) and cholinergic nicotinic receptors (nAChRs) (Freedman et al, 1995; Leonard et al, 1996; Chandy et al, 1998; Glatt et al, 2003; De Luca et al, 2004). Interesting questions for the future are whether NALCN expression or activity is altered by signal transduction events, neuronal plasticity or antipsychotic drugs (Lu et al, 2007). In *C. elegans*, interesting findings with NCA channels have been reported regarding neuronal activity. Loss of NCA channel activity leads to locomotion deficit called fainters, which fail to sustain active locomotion (Humphrey et al, 2007). These ‘‘fainters’’ partially suppress the locomotor, vesicle depletion, and electrophysiological defects of synaptojanin mutants. Suppressor loci include the genes for the NCA ion channels (Humphrey et al, 2007), which are homologs of the vertebrate cation leak channel NALCN (Lu et al, 2007). It was suggested that activation of the NCA ion channel in synaptojanin mutants leads to defects in recycling of synaptic vesicles (Jospin et al (2007). The synaptojanin gene *(SYNJ1)* has been evaluated in bipolar disorder (Saito et al, 2001; Stopkova et al, 2004), and it resides in a susceptibility region for schizophrenia (21q22) (Murtagh et al, 2005; Mirnics et al, 2000). These findings suggest that alterations in NALCN activity may play a role in neuronal plasticity. Together with our results, suggest that the *NALCN* may be involved with the

manifestation of schizophrenia symptoms and associated with antipsychotic-induced TD, but further work is clearly required to confirm this hypothesis.

Disclosure/Conflicts of interest

Mr. Souza, Dr. Romano-Silva, Dr. Zhen and Dr. Wong have nothing to declare. Dr. Lieberman has served as a consultant/ advisor or grantee of Acadia, Astra Zeneca, Bristol-Myers Squibb, Eli Lilly, GlaxoSmithKline, Janssen Pharmaceutica, Lundbeck, Merck, Organon, Pfizer and Wyeth; and holds a patent from Repligen. Dr. Meltzer declares that he is a consultant or grantee of Abbott, Acadia, ARYx, Astra Zeneca, Bristol Myers Squibb, Eli Lilly, Janssen, Memory, Minster, Organon, Pfizer, Solvay, Wyeth, and Vanda. Dr. Kennedy declares that he is a consultant for GlaxoSmithKline.

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Table 1

		Case-control				Clozapine response				Weight gain				Tardive dyskinesia			
		Control		Case		Non-responder		Responder		<7%		≥ 7%		Absent		Present	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
1289356	A	270	61.6	257	60.9	73	50.0	77	57.5	44	57.9	27	46.6	145	56.2	100	61.7
	G	168	38.4	165	39.1	73	50.0	57	42.5	32	42.1	31	53.4	113	43.8	62	38.3
	AA	81	37.0	81	38.4	18	24.7	27	40.3	14	36.8	08	27.6	43	33.3	33	40.7
	AG	108	49.3	95	45.0	37	50.7	23	34.3	16	42.1	11	37.9	59	45.7	34	42.0
	GG	30	13.7	35	16.6	18	24.7	17	25.4	08	21.1	10	34.5	27	20.9	14	17.3
9554752	A	161	36.8	155	36.7	46	31.5	51	38.1	26	34.2	19	32.8	92	35.7	59	36.4
	G	277	63.2	267	63.3	100	68.5	83	61.9	50	65.8	39	67.2	166	63.3	103	63.6
	AA	33	15.1	28	13.3	06	08.2	12	17.9	04	10.5	04	13.8	19	14.7	12	14.8
	AG	95	43.4	99	46.9	34	46.6	27	40.3	18	47.4	11	37.9	54	41.9	35	43.2
	GG	91	41.6	84	39.8	33	45.2	28	41.8	16	42.1	14	48.3	56	43.4	34	42.0
17677552	A	140	32.1	147	34.8	41	28.1	47	35.1	21	27.6	18	31.0	85	32.9	59	34.0
	G	296	67.9	275	65.2	105	71.9	87	64.9	55	72.4	40	69.0	173	67.1	103	66.0
	AA	24	11.0	25	11.8	05	06.8	10	14.9	02	05.3	03	10.3	18	14.0	10	12.3
	AG	92	42.0	97	46.0	31	42.5	27	40.3	17	44.7	12	41.4	49	38.0	35	43.2
	GG	102	46.6	89	42.2	37	50.7	30	44.8	19	50.0	14	48.3	62	48.1	36	44.4
686141	A	140	32.1	127	30.1	47	32.2	30	22.4	25	32.9	13	22.4	72	27.9	49	30.2
	G	296	67.9	295	69.9	99	67.8	104	77.6	51	67.1	45	77.6	186	72.2	113	69.8
	AA	15	06.8	15	07.1	07	09.6	04	06.0	04	10.5	02	06.9	12	09.3	05	06.2
	AG	91	41.6	81	38.4	33	45.2	22	32.8	17	44.7	09	31.0	48	37.2	39	48.1
	GG	113	51.6	115	54.5	33	45.2	41	61.2	17	44.7	18	62.1	69	53.5	37	45.7
12867417	A	91	20.8	78	18.5	37	25.3	26	19.4	22	28.9	10	17.2	58	22.5	46	28.4
	T	347	79.2	344	81.5	109	74.7	108	80.6	54	71.1	48	82.8	200	77.5	116	71.6
	AA	12	05.5	06	02.8	04	05.5	03	04.5	03	07.9	02	06.9	09	07.0	04	04.9
	AT	67	30.6	66	31.3	29	39.7	20	29.9	16	42.1	06	20.7	40	31.0	38	46.9
	TT	140	63.9	139	65.9	40	54.8	44	65.7	19	20.7	21	72.4	80	62.0	39	48.1
658213	A	119	27.3	103	24.5	30	20.5	32	23.9	14	18.4	13	22.4	55	21.3	35	21.6
	C	317	72.7	317	75.5	116	79.5	102	76.1	62	81.6	45	77.6	203	78.7	127	78.4
	AA	14	06.4	14	06.6	03	04.1	05	07.5	00	0.00	01	03.4	09	07.0	04	04.9
	AC	91	41.6	75	35.5	24	32.9	22	32.8	14	36.8	11	37.9	37	28.7	27	33.3
	CC	113	51.6	121	57.3	46	63.0	40	59.7	24	63.2	17	58.6	83	64.3	50	61.7
614728	A	140	32.1	127	30.1	115	78.8	106	79.1	57	75.0	50	86.2	203	21.3	120	25.9
	C	296	67.9	295	69.9	31	21.2	28	20.9	19	25.0	08	13.8	55	78.7	42	74.1
	AA	22	10.0	21	10.0	45	61.6	43	64.2	21	55.3	22	75.9	83	64.3	42	51.9
	AC	96	43.8	85	40.3	25	34.2	20	29.9	15	39.5	06	20.7	37	28.7	36	44.4
	CC	100	45.7	105	49.8	03	04.1	04	06.0	02	05.3	01	03.4	09	07.0	03	03.7
9513851	A	25	05.7	32	07.6	10	06.8	07	05.2	05	06.6	05	08.6	19	07.4	05	02.5
	C	411	94.3	390	92.4	63	93.2	127	94.8	71	93.4	53	91.4	239	92.6	158	97.5
	AA	00	00.0	00	00.0	00	00.0	01	01.5	00	00.0	00	00.0	00	00.0	01	01.2
	AC	25	11.4	32	15.2	10	13.7	05	07.5	05	13.2	05	17.2	19	14.7	02	02.5
	CC	193	88.1	179	84.8	63	86.3	61	91.0	33	86.8	24	82.8	110	85.3	78	96.3
9518807	A	409	93.4	389	92.2	135	92.5	127	94.8	71	93.4	52	89.7	236	92.2	157	96.9
	C	29	6.6	33	7.8	11	07.5	07	05.2	05	06.6	06	10.3	20	07.8	05	03.1
	AA	190	86.8	179	84.8	63	86.3	61	91.0	33	86.8	24	82.8	110	85.3	77	95.1
	AC	29	13.2	31	14.7	09	12.3	05	07.5	05	13.2	04	13.8	18	14.0	03	03.7
	CC	00	00.0	01	00.5	01	01.4	01	01.5	00	00.0	01	03.4	01	00.8	01	01.2

		Case-control				Clozapine response				Weight gain				Tardive dyskinesia			
		Control		Case		Non-responder		Responder		<7%		≥ 7%		Absent		Present	
		n	%	**n**	**%**	n	%	**n**	**%**	n	%	n	%	n	%	n	%
12584031	A	358	81.7	338	80.1	119	81.5	111	82.8	64	84.2	47	81.0	216	83.7	135	83.3
	G	80	18.3	84	19.9	27	18.5	23	17.2	12	15.8	11	19.0	42	16.3	27	16.7
	AA	146	66.7	133	63.0	47	64.4	48	71.6	27	71.1	20	69.0	92	71.3	56	69.1
	AG	66	30.1	72	34.1	25	34.2	15	22.4	10	26.3	07	24.1	32	24.8	23	28.4
	GG	07	03.2	06	02.8	01	01.4	04	06.0	01	02.6	02	06.9	05	03.9	02	02.5
1452112	A	102	23.3	112	26.5	38	26.0	45	33.6	23	30.3	19	32.8	69	26.7	49	30.2
	G	336	76.7	310	73.5	108	74.0	89	66.4	53	69.7	39	67.2	189	73.3	113	69.8
	AA	07	03.2	13	06.2	04	05.5	06	09.0	03	07.9	02	06.9	07	05.4	06	07.4
	AG	88	40.2	86	40.8	30	41.1	33	49.3	17	44.7	15	51.7	55	42.6	37	45.7
	GG	124	56.6	112	53.1	39	53.4	28	41.8	18	47.4	12	41.4	67	51.9	38	46.9
7317836	A	130	29.7	127	30.1	43	29.5	36	26.9	17	22.4	12	20.7	72	27.9	39	24.1
	G	308	70.3	295	69.9	103	70.5	98	73.1	59	77.6	46	79.3	186	72.1	123	75.9
	AA	18	08.2	19	09.0	04	05.5	05	07.5	00	00.0	00	00.0	09	07.0	06	07.4
	AG	94	42.9	89	42.2	35	47.9	26	38.8	17	44.7	12	41.4	54	41.9	27	33.3
	GG	107	48.9	103	48.8	34	46.6	36	53.7	21	55.3	17	58.6	66	51.2	48	59.3
9518320	A	288	65.8	314	74.4	101	69.2	95	70.9	53	69.7	43	74.1	176	68.2	117	72.2
	G	150	34.2	108	25.6	45	30.8	39	29.1	23	30.3	15	25.9	82	31.8	45	27.8
	AA	96	43.8	120	56.9	38	52.1	35	52.2	20	52.6	17	58.6	62	48.1	42	51.9
	AG	96	43.8	74	35.1	25	34.2	25	37.3	13	34.2	09	31.0	52	40.3	33	40.7
	GG	27	12.3	17	08.1	10	13.7	07	10.4	05	13.2	03	10.3	15	11.6	06	07.4
9518331	A	150	34.2	108	25.7	46	31.5	39	29.1	23	30.3	18	31.0	88	34.1	44	37.2
	T	288	65.8	312	74.3	100	68.5	95	70.9	53	69.7	40	69.0	170	65.9	118	72.8
	AA	28	12.8	15	07.1	09	12.3	06	09.0	04	10.5	03	10.3	17	13.2	05	06.2
	AT	94	42.9	78	37.0	28	38.4	27	40.3	15	39.5	12	41.4	54	41.9	34	42.0
	TT	97	44.3	117	55.5	36	49.3	34	50.7	19	50.0	14	48.3	58	45.0	42	51.9
2584531	A	267	61.0	281	66.6	83	56.8	83	61.9	44	57.9	35	60.3	155	60.1	104	64.2
	C	171	39.0	141	33.4	63	43.2	51	38.1	32	42.1	23	39.7	103	39.9	58	33.8
	AA	82	37.4	97	46.0	25	34.2	29	43.3	15	39.5	12	41.4	50	38.8	37	45.7
	AC	103	47.0	87	41.2	33	45.2	25	37.3	14	36.8	11	37.9	55	42.6	30	37.0
	CC	34	15.5	27	12.8	15	20.5	13	19.4	09	23.7	06	20.7	24	18.6	14	17.3
12869164	A	00	00.0	00	00.0	00	00.0	00	00.0	00	00.0	00	00.0	00	00.0	00	00.0
	C	436	100	422	100	146	100	134	100	76	100	58	100	258	100	162	100
	AA	00	00.0	00	00.0	00	00.0	00	00.0	00	00.0	00	00.0	00	00.0	00	00.0
	AC	00	00.0	00	00.0	00	00.0	00	00.0	00	00.0	00	00.0	00	00.0	00	00.0
	CC	218	100	211	100	73	100	67	100	38	100	29	100	129	100.0	81	100.0
3916906	A	158	36.1	126	29.9	52	35.6	45	33.6	25	32.9	20	34.5	94	36.4	46	28.4
	C	280	63.9	296	70.1	94	64.4	89	66.4	51	67.1	38	65.5	167	63.6	116	71.6
	AA	29	13.2	22	10.4	12	16.4	09	13.4	06	15.8	04	13.8	20	15.5	07	08.6
	AC	100	45.7	82	38.9	28	38.4	27	40.3	13	34.2	12	41.4	54	41.9	32	39.5
	CC	90	41.1	107	50.7	33	45.2	31	46.3	19	50.0	13	44.8	55	42.6	42	51.9
9518349	A	262	59.8	232	55.0	95	65.1	81	60.4	53	69.7	39	67.2	161	62.4	93	57.4
	C	176	40.2	190	45.0	51	34.9	53	39.6	23	30.3	19	32.8	97	37.6	69	42.6
	AA	79	36.1	69	32.7	31	42.5	26	38.8	18	47.4	13	44.8	47	36.4	31	38.3
	AC	104	47.5	94	44.5	33	45.2	29	43.3	17	44.7	13	44.8	67	51.9	31	38.3
	CC	36	16.4	48	22.7	09	12.3	12	17.9	03	07.9	03	10.3	15	11.6	19	23.5

		Case-control				Clozapine response				Weight gain				Tardive dyskinesia			
		Control		Case		Non-responder		Responder		<7%		≥ 7%		Absent		Present	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
10508059	A	83	18.9	82	19.4	27	18.8	22	16.4	15	19.7	11	19.0	38	14.7	34	21.0
	G	355	81.1	340	85.0	119	81.5	112	83.6	61	80.3	47	81.0	220	85.3	128	79.0
	AA	07	03.2	08	03.8	03	04.1	04	06.0	04	10.5	01	03.4	07	05.4	03	03.7
	AG	69	31.5	66	31.3	21	28.8	14	20.9	07	18.4	09	31.0	24	18.6	28	34.6
	GG	143	65.3	137	64.9	49	67.1	49	73.1	27	71.1	19	65.5	98	76.0	50	61.7
7328287	A	180	41.1	170	40.5	65	44.5	60	44.8	35	46.1	28	48.3	120	46.5	58	35.8
	G	258	58.9	250	59.5	81	55.5	74	55.2	41	53.9	30	51.7	138	53.5	104	64.2
	AA	39	17.8	31	14.7	14	19.2	16	23.9	09	23.7	06	23.7	29	22.5	09	11.1
	AG	102	46.6	108	51.2	37	50.7	28	41.8	17	44.7	16	44.7	62	48.1	40	49.4
	GG	78	35.6	71	33.6	22	30.1	23	34.3	12	31.6	07	31.6	38	29.5	32	39.5
496238	A	153	34.9	141	33.4	54	37.0	52	38.8	29	38.2	26	44.8	102	39.5	53	32.7
	G	285	65.1	281	66.6	92	63.0	82	61.2	47	61.8	32	55.2	156	60.5	109	67.3
	AA	29	13.2	26	12.3	09	12.3	14	20.9	07	18.4	06	20.7	21	16.3	08	09.9
	AG	95	43.4	89	42.2	36	49.3	24	35.8	15	39.5	14	48.3	60	46.5	37	45.7
	GG	95	43.4	96	45.5	28	38.4	29	43.3	16	48.3	09	31.0	48	37.2	36	44.4
7318529	A	37	8.4	40	9.5	16	11.0	13	09.7	09	11.8	07	12.1	25	09.7	09	05.6
	G	401	91.6	382	90.5	130	89.0	121	90.3	67	88.2	51	87.9	233	90.3	153	94.4
	AA	05	02.3	03	01.4	01	01.4	01	01.5	01	02.6	01	03.4	02	01.6	01	01.2
	AG	27	12.3	34	16.1	14	19.2	11	16.4	07	18.4	05	17.2	21	16.3	07	08.6
	GG	187	85.4	174	82.5	58	79.5	55	82.1	30	78.9	23	79.3	106	82.2	73	90.1
9554772	A	189	43.2	188	44.8	65	44.5	62	46.3	36	47.7	24	41.4	109	22.6	63	38.9
	T	249	56.8	232	55.2	81	55.5	72	53.7	40	52.6	34	58.6	147	57.4	99	61.1
	AA	40	18.3	41	19.4	12	16.4	17	25.4	09	23.7	06	20.7	21	16.4	15	18.5
	AT	109	49.8	106	50.2	41	56.2	28	41.8	18	47.4	12	41.4	67	52.3	33	40.7
	TT	70	32.0	63	29.9	20	27.4	22	32.8	11	28.9	11	37.9	40	31.3	33	40.7
17486808	A	93	21.2	95	22.5	30	20.5	34	25.4	17	22.4	11	19.0	59	22.9	31	19.1
	C	345	78.8	327	77.5	116	79.5	100	74.6	76	77.6	47	81.0	199	77.1	131	80.9
	AA	11	05.0	14	06.6	04	05.5	05	07.5	02	05.3	02	06.9	05	03.9	05	06.2
	AC	71	32.4	67	31.8	22	30.1	24	35.8	13	34.2	07	24.1	49	38.0	21	25.9
	CC	137	62.6	130	61.6	47	64.4	38	56.7	23	60.5	20	69.0	75	58.1	55	67.9
17584161	A	109	24.9	113	26.8	32	21.9	39	29.1	19	25.0	13	22.4	69	26.7	42	25.9
	C	329	75.1	309	73.2	114	78.1	95	70.9	57	75.0	45	77.6	189	73.3	120	74.1
	AA	17	07.8	20	09.5	07	09.6	07	10.4	05	13.2	03	10.3	09	07.0	06	07.4
	AC	75	34.2	73	34.6	18	24.7	25	37.3	09	23.7	07	24.1	51	39.5	30	37.0
	CC	127	58.0	118	55.9	48	65.8	35	52.2	24	63.2	19	65.5	69	53.5	45	55.6
2152324	A	66	15.1	65	15.4	24	16.4	12	09.0	07	09.2	10	17.2	24	09.3	16	09.9
	C	372	84.9	357	84.6	122	83.6	122	91.0	69	90.8	48	82.8	234	90.7	146	90.1
	AA	05	02.3	03	01.4	00	00.0	01	01.8	00	00.0	01	03.4	02	01.6	01	01.2
	AC	56	25.6	59	28.0	24	32.9	10	14.9	07	18.4	08	27.6	20	15.5	14	17.3
	CC	158	72.1	149	70.6	49	67.1	56	83.6	31	81.6	20	69.0	107	82.9	66	81.5

Table 2

SNP	Allele	Frequency	Family	S	E(S)	Var(S)	Z	P
rs1289556	A	0.644	36	40	42.150	14.344	-0.568	0.570
rs9554752	A	0.413	36	30	31.233	10.434	-0.382	0.702
rs17677552	A	0.384	37	30	31.067	11.462	-0.315	0.752
rs614728	A	0.287	30	20	20.667	09.722	-0.214	0.830
rs686141	A	0.277	28	21	20.417	09.160	0.193	0.847
rs12869164	A	0.220	27	21	18.267	08.962	0.913	0.361
rs658213	A	0.744	29	41	40.083	09.410	0.299	0.765
rs9513851	A	0.087	10	05	04.833	02.472	0.106	0.915
rs9518307	A	0.917	10	15	15.167	02.472	-0.106	0.915
rs12584031	A	0.791	26	42	39.217	07.320	1.029	0.303
rs1452112	A	0.289	28	25	19.517	08.650	1.864	0.062
rs7317836	A	0.320	29	22	22.967	09.132	-0.320	0.749
rs9518320	A	0.680	29	41	41.417	09.024	-0.139	0.889
rs9518331	A	0.324	28	20	19.250	08.688	0.254	0.799
rs2584531	A	0.637	29	42	41.583	09.160	0.138	0.890
rs12867417	A	0.000	-	-	-	-	-	-
rs3916906	A	0.348	30	19	19.917	09.410	-0.299	0.765
rs9518349	A	0.517	33	28	32.250	10.465	-1.314	0.188
rs10508059	A	0.195	24	13	17.500	08.528	-1.541	0.123
rs7328287	A	0.435	33	36	31.667	12.614	1.220	0.222
rs496238	A	0.377	32	30	27.500	12.586	0.705	0.481
rs7318529	A	0.088	14	10	07.667	03.722	1.209	0.226
rs9554772	A	0.421	32	34	33.167	12.364	0.237	0.812
rs17486808	A	0.230	24	19	17.250	08.188	0.612	0.540
rs17584161	A	0.269	27	18	19.417	08.660	-0.481	0.630
rs2152324	A	0.179	23	17	15.917	06.660	0.420	0.674

Figure 1

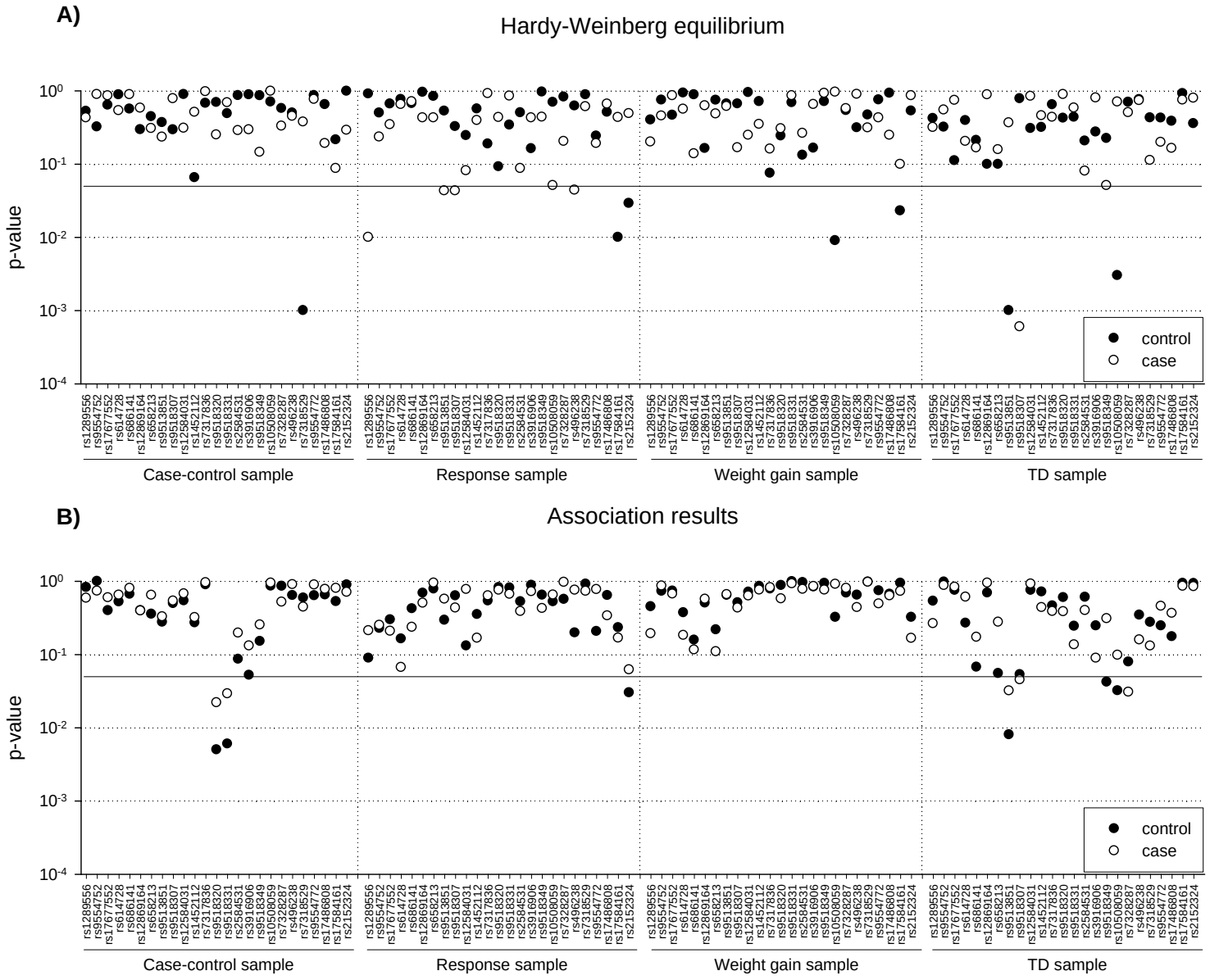
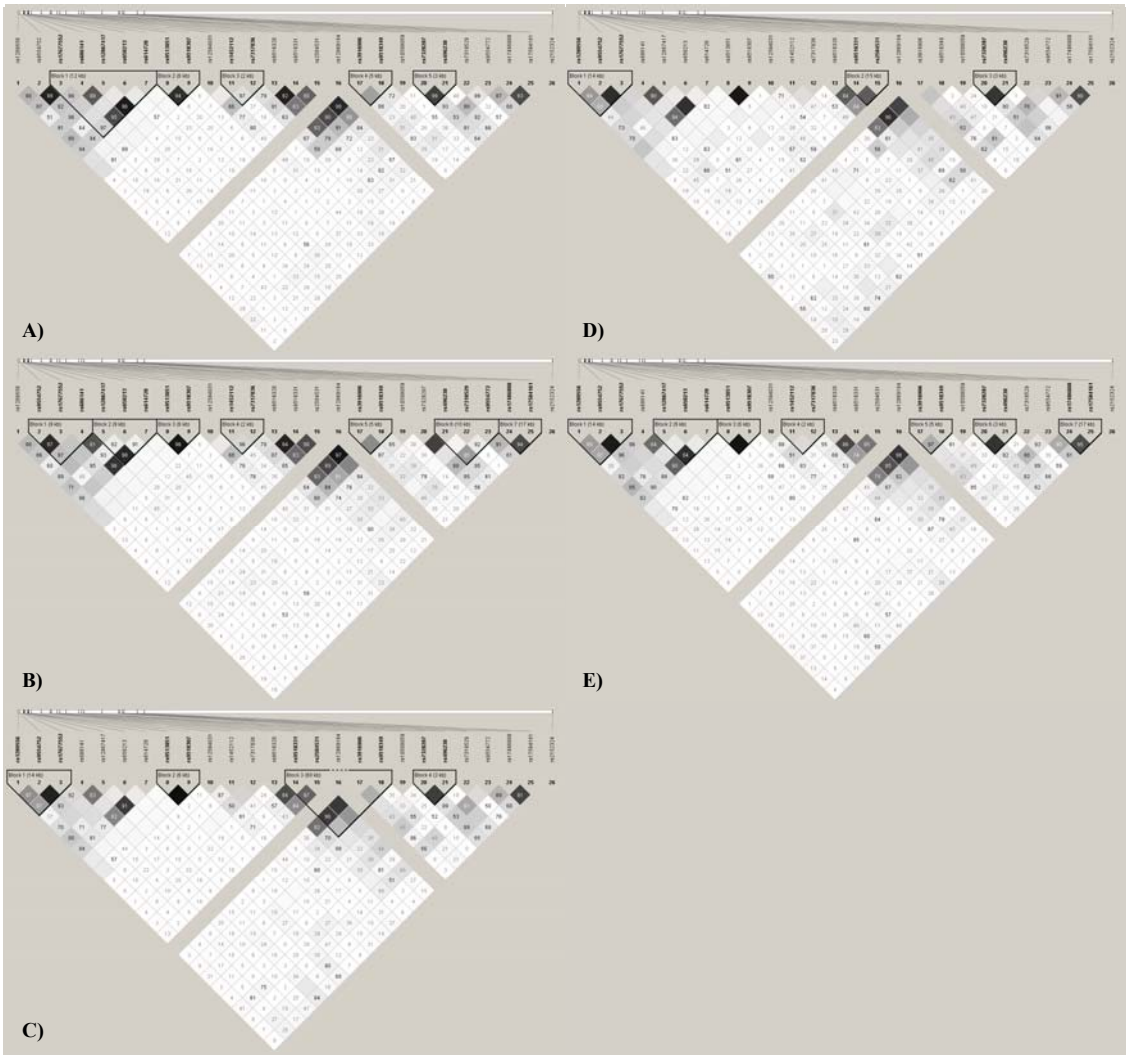

A)
Hardy-Weinberg equilibrium
p-value
control
case
Case-control sample
Response sample
Weight gain sample
TD sample
B)
Association results
p-value
control
case
Case-control sample
Response sample
Weight gain sample
TD sample

Figure S1



Legends

Table 1: Allelic and genotypic frequencies

Table 2: Family–based association test results. S represents the test statistic for observed number of alleles, E represents the expected value of S under null hypothesis and Var(S) represents variance between the observed and expected transmission.

Figure 1: P-values for Hardy-Weinberg equilibrium p-values (A) and association results (B). The solid line represents p = 0.05

Figure S1: LD plot for the analyzed markers in case-control, family, response, weight gain and TD samples (A, B, C, D and E); respectively. Values presented are the D'.

3.5 - Association of antipsychotic induced weight gain and body mass index with GNB3 gene: a meta-analysis

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Association of antipsychotic induced weight gain and body mass index with GNB3 gene: a meta-analysis

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Abstract

It has been reported that C825T variant in the gene encoding the G-protein subunit β3 (GNB3) is associated with antipsychotic-induced weight gain and obesity. We investigated the association of the *GNB3* and antipsychotic-induced weight gain as well as body mass index (BMI) using meta-analytical techniques. Our analysis of 402 schizophrenia subjects showed a trend ($p = 0.072$) only under a fixed-model. As it was observed heterogeneity among the studies ($p = 0.007$), we re-analyzed using a random-effects framework and no significance was found ($p = 0.339$). No evidence for bias publication was reported ($p = 0.868$). Our analysis of 18,903 subjects showed a trend ($p = 0.053$) associating CC and lower BMI under a fixed model. Although no significant association was found, the same pattern (CC and lower antipsychotic-induced weight gain) was observed. Our meta-analysis indicates that firmly establishing the role of pharmacogenetics in clinical psychiatry requires much larger sample sizes that have been reported.

Key words: obesity; BMI; antipsychotic-induced weight gain; GNB-3; meta-analysis

Introduction

Antipsychotic medications are an important component in the medical management of many psychotic conditions. Although them have many notable benefits compared with their earlier counterparts, their use has been associated with reports of dramatic weight gain, diabetes (even acute metabolic decompensation, e.g., diabetic ketoacidosis), and an atherogenic lipid profile (increased LDL cholesterol and triglyceride levels and decreased HDL cholesterol). Because of the close associations between obesity, diabetes, and dyslipidemia and cardiovascular disease, there is heightened interest in the relationship between the antipsychotic drugs and the development of these major cardiovascular disease risk factors (Henderson, 2005).

Search for predictors of drug-related morbidity is becoming increasingly important in persons with major mental illness. Weight gain, glucose and lipid abnormalities are observed more frequently in some novel antipsychotics (Newcomer and Haupt, 2006). The relevance of this side-effect clearly arises from the following considerations: (1) a significant increase in weight gain may affect the compliance to pharmacotherapy and be indirectly responsible for psychosis relapses; (2) weight gain may add to schizophrenia stigma the stigma of obesity and this in turn may lead to poor adherence to the therapy; (3) weight gain may increase the risk for diabetes type II; (4) weight gain can be associated with the metabolic syndrome (Haddad, 2004).

Twenty-nine percent of persons with schizophrenia gain at least 7% of their baseline body weight after being treated with olanzapine in short-term studies (Leucht *et al.* 1999). The FDA has established that weight gain ≥7% from baseline constitutes a clinically meaningful and significant metabolic outcome. Clinical significance of prolonged therapy is now becoming realized as patients develop Type II diabetes mellitus and other obesity-related problems as a consequence of antipsychotic use (American Diabetes Association *et al*. 2004). When looking for possible ethnic differences, clinical studies suggest that Afro-American patients treated with antipsychotics are at higher risk of weight gain (Blin and Micallef, 2001).

G proteins relay signals from each of more than 1000 receptors to many different effectors, including enzymes and ion channels. G proteins are composed of α-subunit that is loosely bound to a tightly associated structure made up of a β subunit and a γ subunit. The activity of the trimeric G protein is regulated by the binding and hydrolysis of guanosine triphosphate by the Gα subunit. The α-subunit to which guanosine diphosphate is bound is inactive and associates with the βγ dimmer (Neves et al. 2002). In 1998, Siffert *et al*. described a C825T polymorphism of the *GNB3* gene. C825T polymorphism is located 1700 bp upstream of the alternative splice site, indicating that affect of GNB3 825T on the splice process is a complex mechanism. Nevertheless, there are examples that single distant nucleotide exchanges, not related to conserved splice branch, donor, and acceptor sites, can cause such alternative splicing (Stallings-Mann *et al.* 1996; Liu *et al.* 1997).

Recent advances in research on the genetic contributions to obesity and its related phenotypes are providing novel tools and targets for the study of mechanisms and risk factors for antipsychotic-induced weight gain. Candidate gene selection should rely on current knowledge on the molecular pathways to weight gain, antipsychotic pharmacokinetics and pharmacodynamics, as well as possible disease-related genetic

links to the side effects under study (Correll and Malhotra, 2004; Muller *et al*. 2004) 825T allele is associated with Gβ3 splice variant, which, despite a deletion of 41 amino acids, is functionally active in reconstituted systems. (Siffert *et al*. 1998). There are few reports linking C825T polymorphism and antipsychotic induced weight gain, that showed no association with either clozapine or olazapine induced weight gain (Tsai *et* al. 2004; Bishop *et al.* 2006). This variant was chosen at it was previously described associated with obesity in several ethnic groups and weight gain during pregnancy (Bishop *et al.* 2006).

Currently available data suggest a worldwide continuous increase in obesity prevalence, which is recently also being observed in developing countries. This prompts some authors to predict an "obesity epidemic" with an increased prevalence of hypertension, stroke, coronary artery disease, and type 2 diabetes mellitus, for which obesity is a major risk factor. Several studies also have investigated whether the 825T allele increases the risk for obesity (Hegele et al. 1999; Siffert et al. 1999a; Siffert et al. 1999b, Stefan *et al.* 2004), these studies have demonstrated that this allele is associated with obesity or increased BMI. Other similar studies, however, have failed to demonstrate this association (Benjafield *et al.* 2001; Hinney *et al.* 2001; Ohshiro *et al.* 2001; Snapir *et al*. 2001; Poston *et al*. 2002; Suwazono *et al.* 2004; Hayakawa *et al.* 2007). Furthermore, it has been evaluated prediction of successful weight reduction under sibutramine therapy and C825T polymorphism but no association was found with no-pharmacologic weight loss strategies (Hauner *et al*. 2003; Potoczna *et al.* 2004). Because of these varying findings, we considered that the apparent association between GNB3 gene variant and obesity or raised BMI had not been demonstrated conclusively. From an epidemiological point of view, we believe that in order to determine the influence of genetic polymorphisms in the occurrence of a specific disease, it is necessary to undertake large-scale studies in the general population. To elucidate better this question we performed this meta-analysis reaching a more significant number of antipsychotic-induced weight gain and BMI related to GNB3 C825T variant.

Methods

Inclusion criteria

Genetic association studies examining the association between C825T and antipsychotic-induced weight gain among patients with schizophrenia that compared the homozygote genotypes were included. Furthermore, in order to analyze the variant influence in the BMI, genetic association studies examining the association between C825T and BMI among adults that compared the homozygote genotypes were also included.

Search strategy

We searched the National Library of Medicine's PubMed online using the search strategy: 'weight gain', 'GNB3', 'obesity' and BMI. This database was searched up to December 2007 looking for terms.

Data extraction

For each study, the following data were extracted using standard forms: author, year of publication, sample ethnicity, case and control sample size, allele frequency, mean age, sex ratio. Ethnicity was coded as European, Asian and African-American.

Statistics

Standardized mean differences (SMDs) and their standard error (S.E.) for individual studies were calculated from 2x2 tables in a case control format. Pooled SMDs were calculated using fixed-effects and random-effects approaches (Der-Simonian and Laird, 1986), and the significance of the pooled SMDs determined using a Z test. The assumption that the effect of allele frequency is constant across studies and between-studies variation is due to random variation was checked using a x2 test for heterogeneity of SMDs. In absence of significant heterogeneity, data were initially analyzed within a fixed-effects framework; otherwise a random-effects framework was employed using Der-Simonian and Laird methods (Der-Simonian and Laird, 1986). This assumes that between-study variation is due to both random variation and an individual study effect. Random-effects models are more conservative and generate a wider confidence interval. Publication bias was assessed by means of a funnel plot of individual study log SMD against S.E. log SMD, and formally by the method of Egger (Egger et al. 1997), which is based on a weighted linear regression of standard normal deviation of the SMD (standardized effect) on the inverse of the standard error of the SMD (precision). Data were analyzed using the STATA version 8.0 statistical software package (Stata Corporation, College Station, TX, USA).

Results

Antipsychotic-induced weight gain

A total of five studies published between 2004 and 2007, comprising five independent samples, were identified by the search strategy, met the inclusion criteria and contributed to the meta-analysis. Two studies (De Luca et al. 2004; Souza et al. 2007) included in the meta-analysis are from our group and they were published in the conferences abstract book. Each sample was included independently in the analysis. The main outcome investigated in this meta-analysis was the weight percentage change in kilograms after the treatment. To dissect the genotype effect only the CC and TT genotypes were included in the analysis. The data entered in the meta-analysis table were mean, standard deviation and number of subjects for CC and TT genotype respectively. Two were performed with Caucasian subjects, two with Asian (Chinese) and one with mixed population (Caucasian and African-American in Souza *et al.* 2007) (Table 1). When all samples were included there was a trend for CC association with lower antipsychotic-induced weight gain under a fixed model (z=1.80, p=0.072, SMD 0.27, 95% CI –0.025 – 0.584); however, there was a significant heterogeneity between the studies (X_2= 14.25, d.f.=4, p=0.007). As it was found heterogeneity in this sample, this data set has been analyzed using random effects model as it is the most appropriate model. When the analysis was re-run within a random-effects framework no significance was found (z=0.96, p=0.339, SMD 0.28, 95% CI -0.03 - 0.58) (Figure 1A). As there are strong evidences that ethnic background is an important confounding factor in antipsychotic-

induced weight gain genetics, stratified analysis by ethnicity were performed. Asians and Caucasians subpopulations (as there is just one study that analyzed African-American population) were created and their results did not show significant associations (data not shown). A Begg's funnel plot with 95% confidence limits is presented in Figure 1B. Egger's test did not report evidence of publication bias (intercept= 0.784, t=0.18, p=0.868, 95% CI –12.973 – 14.542) (Figure 1C).

BMI association

A total of 39 studies published between 1999 and 2007, comprising 34 independent samples, were identified by the search strategy, met the inclusion criteria and contributed to the meta-analysis. Five studies that reported the BMI value for one of the homozygous genotypes grouped with the heterozygous were excluded. 16 studies did not report BMI for each genotype and another one that reported it for each allele were also excluded. From the 18 studies that reported BMI for CC and TT groups, 12 were performed with Caucasian subjects, three with Asian (Chinese or Japanese), one with African-American and another two with mixed populations (Caucasian and Asian in Siffert *et al*. 1999b; Caucasian and African-American in Danoviz *et al.* 2006) (Table 2). When all samples were included there was a trend for association of CC and lower BMI under a fixed model (z=1.93, p=0.053, SMD 0.05, 95% CI 0.00 - 0.09), and there was also a trend of significant heterogeneity between the studies (X_2= 31.70, d.f.=21, p=0.063) (Figure 2A). A Begg's funnel plot with 95% confidence limits is presented in Figure 2B. This illustrates a certain asymmetry with predominance of small and positive studies over small and negative studies. Egger's test did not indicate evidence of publication bias (intercept= 0.897, t=1.52, p=0.143, 95% CI –0.331 – 2.125), although it is possible to notice that small samples with low precision having a large standardized effect and large samples with high precision having small-standardized effect (Figure 2C).

Discussion

Weight gain is probably the most actual side effect in antipsychotic treatment due to the wide use of new antipsychotics, on the other hand the pharmacogenetic of antipsychotics has focused more on side effects like tardive dyskinesia (Lerer et al. 2005), and therefore we were able to find only a few studies exploring weight gain. Our analysis of schizophrenia subjects showed a trend (p = 0.072) only under a fixed-model probably because we included only the homozygous CC and TT in the analysis however the overall sample was composed by 406 subjects. As it was observed heterogeneity among the studies (p = 0.007), we re-analyzed using a random-effects framework and no significance was found (p = 0.339). No evidence for bias publication was reported (p = 0.868). However, there are few published studies in the area and a much larger sample size would be needed to test this hypothesis definitively.

A considerable part of obesity is due to environmental factors and lifestyle, but between 40-70% of the variation of body mass index (BMI) is estimated to be heritable (Comuzzie *et al*. 1988). Our analysis of 18,903 subjects showed a trend (p = 0.053) associating CC and lower BMI under a fixed model. Even in the BMI analysis we selected only the CC and TT subjects, this can be an advantage since we reduced the

genetic heterogeneity, however this reduces the statistical power of the sample. There may be a trend towards an association but the size of the effect is small and much larger studies are needed to demonstrate this association. Although no significant association was found, the same pattern (CC and lower antipsychotic-induced weight gain) was observed.

The 825T allele has already been analyzed in various contexts that could increase risk for phenotypes metabolic syndrome-related. Total cholesterol is significantly higher in subjects with the T allele among Japanese (Ishikawa *et al.* 2000); and this same allele was associated with end-stage renal disease in type 2 diabetes mellitus (Gumprecht *et al.* 2001). The C825T role in hyperlipidemia, diabetes, and diabetic complications have been controversial (Fogarty *et al.* 1998; Siffert et al. 1999; Rosskopf *et* al. 2000; Beige *et al.* 2000; Zychma *et al.* 2000; Gumprecht *et al.* 2001; Shcherbak *et al.* 2001; Hanon *et al.* 2002; Dzida *et al.* 2002; Von Beckerath *et al.* 2003; Brand *et al.* 2003; Yamamoto *et al.* 2004; Andersen *et al.* 2006; Hayakawa *et al.* 2007). At the same way, no conclusive data have been published in studies that evaluated genetic association of C825T with antipsychotic-induced weight gain (De Luca *et al.* 2004; Tsai *et al*. 2004; Wang *et al.* 2005; Bishop *et al*. 2006; Souza *et al.* 2007).

Metabolic imbalance has been found more common in drug-naïve schizophrenics rather than general population (Ryan and Thakore, 2002) thus this strategy to combine studies that have focussed on obesity and the side-effect of schizophrenia treatment analyzing common genetic targets seems very intriguing because it can help to uncover the common genetic background. This meta-analysis pointed out that the number of pharmacogenetic studies of antipsychotic-induced weight gain is very small and sometimes the sample size is not adequate. Furthermore, for some studies, position and dispersion measures are not always specified for both genotype groups and it is very important to specify whether SD or SEM has been described for including the study in future meta-analysis.

On the other hand the phenotype BMI that is closely related to weight gain has been widely investigated in regards of the C825T. This discrepancy is due to the fact that baseline BMI can be measured as a cross-sectional assessment instead weight change requires follow-up with the risk to lose subjects. Therefore, we suggest some points for the pharmacogenetics studies in order to enhance the power of antipsychotic-induced weight gain meta-analysis studies: to select phenotype such as the presence of metabolic syndrome that is measurable in a cross-sectional assessment; use of larger sample size; controlling for ethnicity and previous antipsychotic exposure.

Conclusion

In this paper we suggest a new way how to apply the meta-analytic technique to genetic association studies for dissecting the genetic influence in related phenotypes to show possible bias in the published studies and suggesting different methodological approaches to improve the overall quality of pharmacogenetic studies of antipsychotic-induced weight gain.

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Table 1

Study	Year	n	Ancestry	Subjects
De Luca *et al.*	2004	80	Caucasian	Schizophrenia patients weight-gain after 6 weeks taking clozapine
Tsai *et al.*	2004	87	Asian	Schizophrenia patients weight-gain after 4 months taking clozapine
Wang *et al.*	2005	134	Asian	Schizophrenia patients weight-gain after 13 months taking clozapine
Bishop *et al.*	2006	42	Caucasian	Schizophrenia patients weight-gain after 6 weeks taking olanzapine
Souza *et al.*	2007	59	Caucasian/ African-American	Schizophrenia patients weight-gain after 14 weeks taking mixed antipsychotics

Table 2

Study	Year	n	Ancestry	Subjects
Siffert	1999	277/960	Caucasian/Asian	Healthy male
Hegele	1999	213	Caucasian	Randomly selected individuals
Siffert	1999	197	Caucasian	Hypertensive individuals
Hengstenberg	2001	2052/606	Caucasian	Randomly selected/ Myocardial infarction
Snapir	2001	903	Caucasian	Randomly selected males
Poston	2001	175	African-American	Randomly selected individuals
Hanon	2002	306	Caucasian	Atherosclerosis Prevention Clinic subjects
Brand	2003	1512	Caucasian	Randomly selected
Huang	2003	1165	Asian	Case-control hypertension study
Beckerath	2003	1338	Caucasian	Case-control coronary artery disease study
Sartori	2003	461	Caucasian	Hypertensive individuals
Stefan	2004	774	Caucasian	Case-control impaired glucose tolerant study
Potoczna	2004	304	Caucasian	Severely obese individuals
Yamamoto	2004	806	Asian	Subject who participated in a medical check-up
Martin	2005	144	Caucasian	Case-control hypertension study
Danoviz	2006	1568	Caucasian/African-American	Randomly selected individuals
Andersen	2006	4387	Caucasian	Glucose tolerant individuals
Hayakawa	2007	755	Asian	Case-control diabetes study

Figure 1

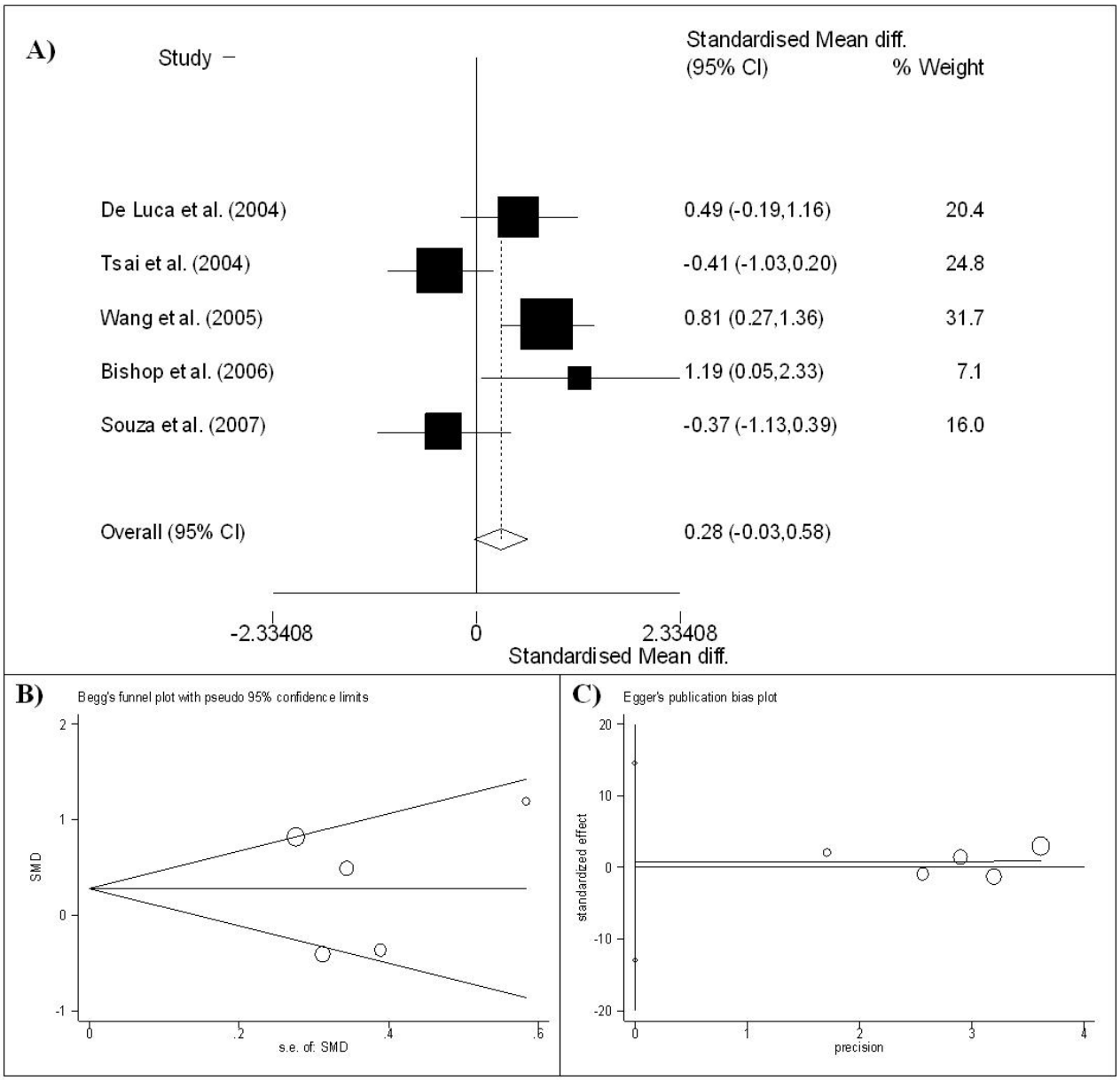
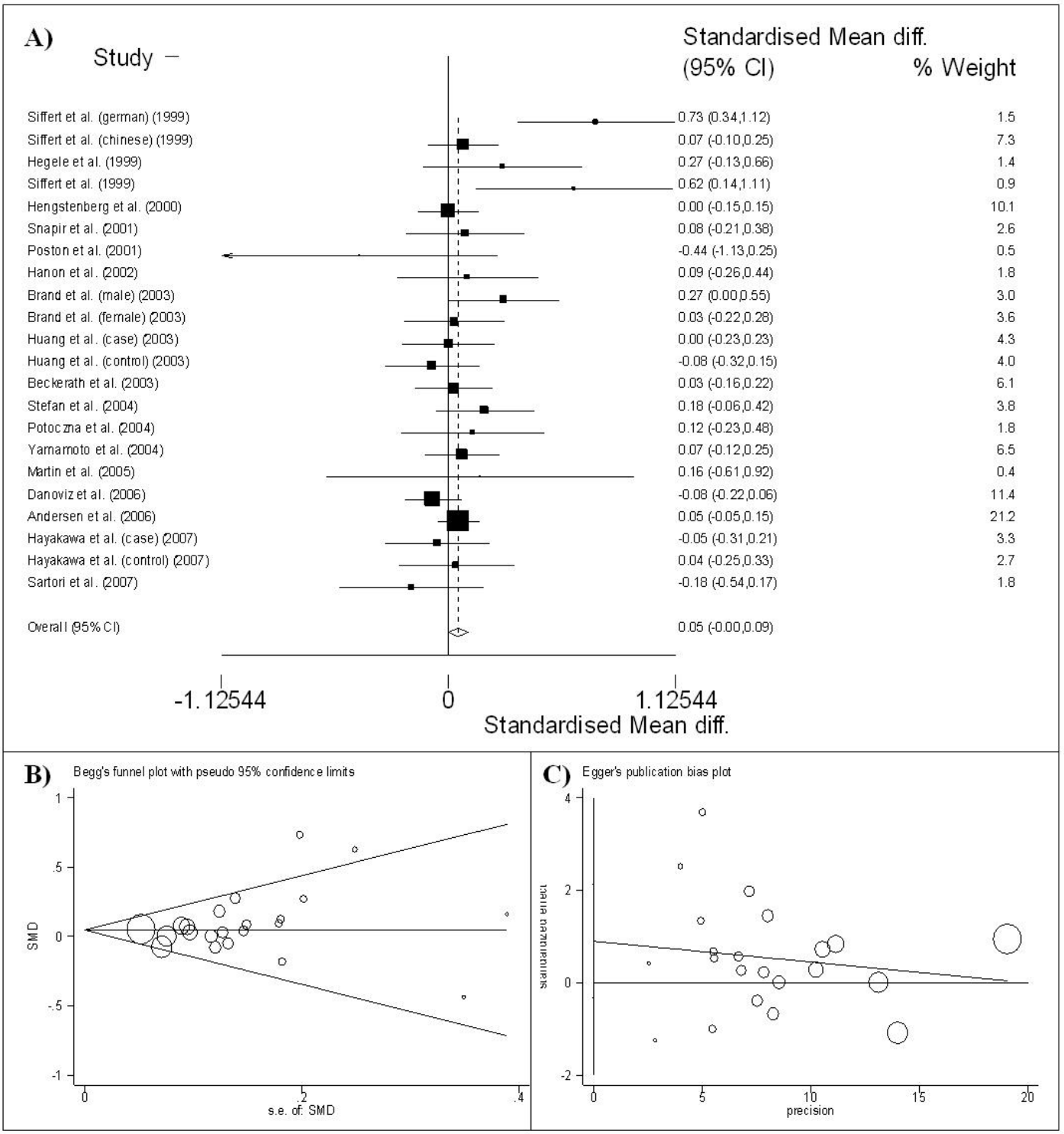

A)
Study
Standardised Mean diff.
(95% CI)
% Weight
De Luca et al. (2004)
0.49 (-0.19,1.16)
20.4
Tsai et al. (2004)
-0.41 (-1.03,0.20)
24.8
Wang et al. (2005)
0.81 (0.27,1.36)
31.7
Bishop et al. (2006)
1.19 (0.05,2.33)
7.1
Souza et al. (2007)
-0.37 (-1.13,0.39)
16.0
Overall (95% CI)
0.28 (-0.03,0.58)
-2.33408
0
2.33408
Standardised Mean diff.
B)
Begg's funnel plot with pseudo 95% confidence limits
SMD
s.e. of: SMD
C)
Egger's publication bias plot
standardized effect
precision

Figure 2


A)
Study
Standardised Mean diff.
(95% CI)
% Weight
Siffert et al. (german) (1999) 0.73 (0.34,1.12) 1.5
Siffert et al. (chinese) (1999) 0.07 (-0.10,0.25) 7.3
Hegele et al. (1999) 0.27 (-0.13,0.66) 1.4
Siffert et al. (1999) 0.62 (0.14,1.11) 0.9
Hengstenberg et al. (2000) 0.00 (-0.15,0.15) 10.1
Snapir et al. (2001) 0.08 (-0.21,0.38) 2.6
Poston et al. (2001) -0.44 (-1.13,0.25) 0.5
Hanon et al. (2002) 0.09 (-0.26,0.44) 1.8
Brand et al. (male) (2003) 0.27 (0.00,0.55) 3.0
Brand et al. (female) (2003) 0.03 (-0.22,0.28) 3.6
Huang et al. (case) (2003) 0.00 (-0.23,0.23) 4.3
Huang et al. (control) (2003) -0.08 (-0.32,0.15) 4.0
Beckerath et al. (2003) 0.03 (-0.16,0.22) 6.1
Stefan et al. (2004) 0.18 (-0.06,0.42) 3.8
Potoczna et al. (2004) 0.12 (-0.23,0.48) 1.8
Yamamoto et al. (2004) 0.07 (-0.12,0.25) 6.5
Martin et al. (2005) 0.16 (-0.61,0.92) 0.4
Dan oviz et al. (2006) -0.08 (-0.22,0.06) 11.4
Andersen et al. (2006) 0.05 (-0.05,0.15) 21.2
Hayakawa et al. (case) (2007) -0.05 (-0.31,0.21) 3.3
Hayakawa et al. (control) (2007) 0.04 (-0.25,0.33) 2.7
Sartori et al. (2007) -0.18 (-0.54,0.17) 1.8
Overall (95% CI) 0.05 (-0.00,0.09)
-1.12544
0
1.12544
Standardised Mean diff.
B)
Begg's funnel plot with pseudo 95% confidence limits
SMD
s.e. of: SMD
C)
Egger's publication bias plot
standardized effect
precision

Legends

Table 1: Characteristics of included sample in the antipsychotic-induced weight gain analysis

Table 2: Characteristics of included sample in the BMI analysis

Figure 1: Meta-analysis results of GNB3 C825T association with antipsychotic-induced weight gain. A) Forest plot of studies assessing the effect of CC variant for lower weight gain: overall genotype effect for dichotomous outcomes. Standardized mean difference (SMD)>1 in favour CC effect in lower weight gain; SMD<1 against CC effect in lower weight gain. B) Begg's funnel plot of publication bias with pseudo 95% confidence limits. C) Egger's publication bias plot.

Figure 1: Meta-analysis results of GNB3 C825T association with BMI. A) Forest plot of studies assessing the effect of CC variant for lower BMI: overall genotype effect for dichotomous outcomes. Standardized mean difference (SMD)>1 in favour CC effect in lower BMI; SMD<1 against CC effect in lower BMI. B) Begg's funnel plot of publication bias with pseudo 95% confidence limits. C) Egger's publication bias plot.

4. Conclusões

Após a análise das 90 variantes mapeados nos genes GSK-3β, GFRα1-4, NALCN e GNB-3, uma análise individual destes marcadores sugere que:

- GSK-3β: três variantes (rs7624540, rs4072520 e rs6779828) mostraram genótipos associados com esquizofrenia, sendo que o rs4072520 permanece significativo após correção por 1.000 permutações. Não fora encontrada associação com a esquizofrenia em nossa amostra de famílias e com a resposta ao tratamento com clozapina.

- GFRα1-4: GFRα1 rs11197557 foi associado com a esquizofrenia na amostra de caso-controle pareado e rs730357, bem como alguns haplótipos, mostraram um padrão de transmissão alterado. Embora nenhum dos marcadores em GFRα1 esteve associado com a resposta ao tratamento de forma individual, dois haplótipos (rs11197612-rs3781514 e rs12413585-rs730057-rs1197612) o estiveram. No gene GFRα2, três variantes (rs1128397, rs13250096 e rs4567028) e alguns haplótipos mostram-se associados com a resposta ao tratamento. No gene GFRα3, o rs11242417 e o haplótipo contendo todos os quatro marcadores analisados foram associados com a susceptibilidade à esquizofrenia. Nenhuma associação fora encontrada no gene GFRα4.

- GPX e MnSOD: não foram encontradas associações com a resposta ao tratamento com clozapina nem com a gravidade de sintomas.

- NALCN: na amostra caso-controle foram encontradas associações nos marcadores rs9518320 e rs9518331 e nos haplótipos compostos por rs7317836, rs9518320, rs9518331, rs2584531 e rs3916906. A amostra de famílias não apresentou nenhuma associação significativa. Uma variantes, rs2152324, esteve associada com a resposta ao tratamento, enquanto um haplótipo formado por rs10508059-rs7328287-rs496238 for a associado ao ganho de peso induzido por clozapina. Cinco variantes foram associadas de maneira individual com a TD (rs9513851, rs9518307, rs9518349, rs10508059 e rs7328287) e dois haplótipos (rs9513851-rs9518307 e rs7328287-rs496238).

- GNB-3: a meta-análise mostrou que a variante CC está associada com um menor índice de massa corpórea, bem como com menor ganho de peso, embora não se alcance significância estatítisca nessa observações (p = 0,339 e p = 0,053).

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