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DANIEL MARTINS DE SOUZA

"DETERMINAÇÃO DO PERFIL DE EXPRESSÃO GÊNICA E PROTEÔMICA EM TECIDO CEREBRAL DE PACIENTES ESQUIZOFRÊNICOS."

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) DANIEL MARTINS DE SOUZA e aprovada pela Comissão Julgadora.

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Orientador: Emmanuel Dias-Neto

Co-orientador: Prof. Dr. José Camillo Novello

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BANCA EXAMINADORA

Prof. Dr. Emmanuel Dias Neto (Orientador) _____
Assinatura

Prof. Dr. Gilberto Barbosa Domont _____
Assinatura

Prof. Dr. Francesco Langone _____
Assinatura

Prof. Dr. Gonçalo Amarante Guimarães Pereira _____
Assinatura

Prof. Dr. Paulo Clemente Sallet _____
Assinatura

Prof. Dr. Marcos Nogueira Eberlin _____
Assinatura

Prof. Dr. Orestes Vicente Forlenza _____
Assinatura

Prof. Dr. Paulo Sérgio Lopes de Oliveira _____
Assinatura

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O Conceito desta da tese:

Cremos que o objetivo de uma tese deva ter como foco um significado especial para a humanidade, independente do tamanho da contribuição. Pensamos que ao estudar a esquizofrenia, poderíamos auxiliar de alguma maneira uma melhor qualidade de vida para as pessoas afetadas e suas famílias.

Este foi o principal motivo que nos moveu nestes 4 anos a buscar nossos objetivos.

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Abreviaturas:

* 2-DE: Eletroforese de Duas Dimensões em Gel de Poliacrilamida
* 5-HIAA: Ácido 5-Hidroxiindolacético
* 5-HT: Serotonina
* CBB: *Coomassie Brillant Blue* (Azul Brilhante de Coomassie)
* DA: Dopamina
* DIGE: *Difference Gel Electrophoresis* (Gel de Eletroforese Diferencial)
* DLPFC: Córtex Pré-Frontal Dorsolateral
* EM: Espectrômetro de Massas
* EMT: Estimulação Magnética Transcraniana
* EST: *Expressed Sequenced Tags* (Etiquetas de Sequências Expressas)
* fMRI: Imagem por Ressonância Magnética Funcional
* GABA: Ácido Gama-Aminobutírico
* GIST: *Global Internal Standard Technology* (Tecnologia de Padrão Interno Global)
* GLU: Glutamato
* ICAT: *Isotope-Coded Affinity Tags* (Etiquetas Isotópicas de Afinidade)
* ICPL: *Isotope-Coded Protein Labeling* (Rotulação de Proteínas com Etiquetas Isotópicas)
* IEF: Isoeletrofocalização ou Focalização Isoelétrica
* iTRAQ: *Isobaric Tags for Relative and Absolute Quantification* (Etiquetas Isobáricas Para Quantificação Relativa e Absoluta)
* LSD: Ácido D-Lisérgico
* MALDI-TOF: *Matrix-Assisted Laser Desorption/Ionization-Time of Flight* (Desorção e Ionização por Laser Assistida por Matriz - Tempo de Vôo)
* MRI: Imagem por Ressonância Magnética
* MS: Espectrometria de Massas
* MS/MS: Espectrometria de Massas em Série
* MW: Peso Molecular
* NPC: Neurônios Piramidais Corticais
* pI: Ponto Isoelétrico
* PMF: *Peptide-Mass Finger Printing* (Impressão Digital das Massas de Peptídeos)
* PSD: "*Post-Source Decay*"
* qPCR: Reação em Cadeia da Polimerase em Tempo Real
* RT-PCR: Reação em Cadeia da Polimerase por Transcriptase Reversa
* SAGE: *Serial Analysis of Gene Expression* (Análise Seriada da Expressão Gênica)
* SCZ: Esquizofrenia
* SNC: Sistema Nervoso Central

Resumo

A esquizofrenia é um distúrbio mental debilitante que afeta aproximadamente 1% da população mundial, caracterizado por sintomas produtivos como delírios e alucinações e sintomas negativos como apatia e decréscimo das emoções. Nesta tese, realizamos estudos do transcriptoma e do proteoma em tecido cerebral de pacientes com esquizofrenia, buscando identificar genes e proteínas envolvidas com esta doença.

Em nossas análises transcricionais, utilizamos a técnica de *Serial Analysis of Gene Expression* (SAGE), uma abordagem ainda inédita em esquizofrenia. Os dados permitiram a análise de mais de 20 mil transcritos, e apontaram para o possível envolvimento de genes associados a processos como mielinização, função sináptica, metabolismo energético e homeostase de cálcio, incluindo genes anteriormente envolvidos com esquizofrenia, e também uma boa parcela de genes até então não associados com a doença. Uma pequena fração destes novos marcadores foi avaliada por *Real-Time* PCR permitindo a confirmação de alguns achados de SAGE.

Nossas análises de proteoma foram feitas com as técnicas de eletroforese de duas dimensões, seguida por espectrometria de massas e pela técnica de *shotgun proteomics*, também inédita em esquizofrenia. Estas análises foram realizadas com amostras de diferentes regiões cerebrais, incluindo córtex pré-frontal e lobo temporal anterior, e apontaram para alterações quantitativas em proteínas relacionadas com a homeostase de cálcio, citoesqueleto, metabolismo energético e de oligodendrócitos.

De modo geral, observamos uma boa consistência entre os resultados obtidos quando estudamos diferentes classes de marcadores potenciais (genes e proteínas). Por muitas vezes os genes alterados não corresponderam a alterações quantitativas nas mesmas proteínas, no entanto, na maior parte dos casos, observamos alterações consistentes nas mesmas vias. Observamos a regulação diferencial do metabolismo de oligodendrócitos, energético, sináptico e revelamos a provável alteração da homeostase de cálcio em cérebros de pacientes com esquizofrenia, além de identificarmos genes e proteínas diferencialmente expressas nunca relacionadas à doença.

Nossos dados reforçam achados prévios, apontam potenciais biomarcadores e podem fornecer novas pistas na compreensão da esquizofrenia.

Abstract:

Schizophrenia is a mental debilitating disorder that affects 1% of the world population. It is characterized by positives symptoms such as delirium and hallucinations and negative symptoms such as apathy and emotion decrease. Here, we have studied the transcriptome and proteome of brain samples of patients with schizophrenia, in an attempt to identify genes and proteins markers of the disease.

Our transcriptome analyses of pre-frontal cortex were performed with Serial Analysis of Gene Expression (SAGE), here used for the first time in the study of schizophrenia. The data obtained allowed the analysis of approximately 20,000 transcripts, which suggested the importance of myelinization, synaptic function, energy metabolism and calcium homeostasis, in the genesis of schizophrenia. A series of genes previously implicated in the disease were identified, together with new potential markers which were revealed here for the first time. A small fraction of these was validated using real-time PCR, which confirmed some of the SAGE findings.

Two-dimensional gel electrophoresis, mass spectrometry and shotgun proteomics were the approaches used here for large-scale protein analysis in schizophrenia. These approaches were used in brain samples derived from distinct areas such as pre-frontal cortex and anterior temporal lobe, and indicated quanitative alterations of proteins involved with calcium homeostasis, energy and oligodendrocyte metabolism and cytoskeleton.

In general, a good correlation was observed when the different approaches (transcriptome and proteome) were used. In may cases, the alterations of some genes was not reflected by a correspondent alteration of the encoded protein. However, in most cases, reproducible alterations were found in the same pathways. Beside the identification of new schizophrenia-related genes and proteins, we also confirmed the the differential regulation of oligodendrocyte, synaptic and energetic metabolism, in this disease.

Our data reinforce previous findings, and suggest new potential biomarkers that may contribute to the understanding of schizophrenia.

Introdução Geral

1.1 Esquizofrenia

No final do século XIX, em 1899, o psiquiatra alemão Emil Kraepelin retratou na 6ª edição de sua obra "Tratado de Psiquiatria" um distúrbio mental orgânico, o qual nominou *"Dementia praecox"* (Demência Precoce), que consistia na deterioração progressiva e persistente da capacidade mental de pessoas jovens[1,2]. Este mesmo distúrbio foi mais tarde redescrito com maiores detalhes e reclassificado por Eugene Bleuler como "Esquizofrenia"[3] (etimologicamente, fenda na mente) devido à fragmentação entre o pensamento e as emoções.

1.1.1 Características Clínicas e Epidemiológicas

A esquizofrenia (SCZ) é uma doença mental crônica, debilitante e estritamente humana que se manifesta em aproximadamente 1% da população mundial, sendo homens e mulheres igualmente afetados[4]. As pessoas acometidas têm sua capacidade intelectual, social e produtiva comprometida, o que causa grande impacto psicológico e afetivo em suas famílias, além do alto custo com tratamento e hospitalização.

A SCZ é caracterizada como uma doença multifatorial desencadeada pela ação conjunta de fatores exógenos (ou ambientais) como infecções virais severas[5,6,7], complicações obstétricas[8,9], luto materno durante a gestação revisado em [10], uso de álcool e drogas[11], e a vida conturbada nos grandes centros

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urbanos[12,13,14] somados a fatores endógenos, como pré-disposição genética [revisado em 15], a qual é um dos grandes determinantes do desencadeamento da SCZ[16,17].

A SCZ começa a manifestar-se normalmente em jovens adultos (entre 16 e 30 anos), sendo os sintomas produtivos principais os delírios e as alucinações. Alucinações são percepções sem correspondente estimulo externo. Na SCZ as alucinações auditivas são as mais freqüentes. Os delírios são caracterizados clinicamente pela crença em idéias ou fatos não-reais, como por exemplo, a crença do paciente de que existem conspirações contra ele ou que existem pessoas perseguindo ou observando seu comportamento. Muitas vezes o delírio esta por trás de um comportamento social bizarro apresentado pelos pacientes. Além destes sintomas produtivos, os chamados "sintomas negativos" também são comuns e incluem a incapacidade de concentração, perda da sensação de satisfação, perda da disposição, desorganização ou empobrecimento dos pensamentos e da fala, diminuição do afeto e isolamento social[18]. Os sintomas produtivos e negativos podem oscilar ao longo do tempo, podendo o paciente apresentar predominantemente um ou mais sintomas num determinado período. A combinação dos diferentes sintomas classifica a SCZ em subtipos, como mostra a Tabela 1.

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Tabela 1: Subtipos de esquizofrenia e seus principais sintomas.

Subtipo de Esquizofrenia	*Principais Sintomas*
Paranóide	Marcada pelos sintomas produtivos e pela presença de delírios (perseguição e conspiração contra o paciente), alucinações auditivas e ansiedade.
Residual (ou Simples)	Marcada pelos sintomas negativos. Não apresenta sintomas positivos (difícil diagnóstico). Afastamento e inadequação social e afetiva (parece não gostar das pessoas que ama), pensamentos ilógicos. Destruição da personalidade em longo prazo.
Desorganizada (ou Hebefrênica)	Mesmos sintomas da residual, porém com maior desordem nos pensamentos. Os pacientes não têm objetivos a seguir e não conseguem se concentrar em atividades como leitura ou trabalho.
Catatônica	Alterações na psicomotricidade: excitação, posturas socialmente inadequadas, estupor e rigidez. Medo e alucinações podem motivar a rigidez. Pode haver a perda de alguns movimentos até a total imobilização. Negativismo (resistência a ordens).
Indiferenciada	A combinação dos sintomas de mais de um subtipo ou sintomas que não podem ser classificados nos outros subtipos.

1.1.1.1 Diagnóstico

O diagnóstico da SCZ, estritamente clínico, é baseado, sucintamente, na presença concomitante significativa de pelo menos dois dos sintomas supracitados por no mínimo seis meses[18]. O comportamento criminal *per se* não é concomitante da SCZ, mas os pacientes podem cometer atos violentos em

resposta às alucinações ou delírios ou ainda devido às frustrações nas interações sociais[19]. A prevalência de suicídio entre os pacientes está ao redor de 10%[20].

1.1.1.2 Tratamento

O tratamento da SCZ é essencialmente medicamentoso. O controle das manifestações clínicas é centrado nos neurotransmissores que por sua vez atuam controlando as respostas neuronais.

Sucintamente, neurônios piramidais corticais (NPC) são regulados primordialmente por interneurônios inibitórios pré-sinápticos através de receptores de ácido gama-aminobutírico (GABA). Ambos, NPC e interneurônios são controlados por dopamina (DA), serotonina (5-HT) e glutamato (GLU). Os NPC processam e armazenam informações e o comprometimento de sua regulação, principalmente uma resposta excessiva destes, evidentemente compromete suas funções, causando os sintomas observados na SCZ[21].

O primeiro medicamento usado no tratamento da SCZ foi a Clorpromazina, uma droga inicialmente usada como anti-histamínico, mas que demonstrou ter atividade antipsicótica em pacientes com SCZ[22]. Pesquisas posteriores demonstraram que o mecanismo de ação da clorpromazina se dava pelo bloqueio de receptores dopaminérgicos e a partir deste dado, outros medicamentos foram desenvolvidos a partir de antagonistas dopaminérgicos[23]. Novos dados sugeriram ainda que o alvo destes "neurolépticos de primeira geração" ou "neurolépticos típicos" [24] era preferencialmente o receptor de dopamina tipo D2[25]. Cerca de 20% dos pacientes tratados com estas drogas apresentam remissão total dos sintomas: o restante apresenta resposta parcial ao medicamento[26]. O maior problema deste

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tratamento é a sua baixa eficácia em relação aos sintomas negativos da SCZ[27]. O principal efeito colateral do tratamento com neurolépticos de primeira geração é o distubio motor já que os receptores D2 de dopamina dos neurônios motores no núcleo da base são também afetados[23]. Outros efeitos colaterais estão descritos na Tabela 2.

Os "neurolépticos de segunda geração" ou "neurolépticos atípicos" foram desenvolvidos nos últimos trinta anos, com o objetivo de aumentar o efeito terapêutico, incluindo a melhor eficácia quanto aos sintomas negativos e reduzir os efeitos colaterais destas drogas[24]. Estes medicamentos ligam-se com menor afinidade aos receptores de dopamina D2, D1 e D4, e atuam também em outras vias como a glutamatérgica e serotonérgica[28]. A eficácia do tratamento com neurolépticos atípicos em relação aos sintomas positivos e negativos é similar ou pouco superior se comparada aos neurolépticos típicos, porém, os efeitos extrapiramidais são reduzidos[29,30,31] bem como o risco de discinesia tardia, ou seja, movimentos involuntários que continuam mesmo após o término da administração do medicamento[32]. Os efeitos colaterais dos medicamentos usados no tratamento da SCZ estão descritos na Tabela 2.

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Tabela 2: Os medicamentos para tratamento da esquizofrenia e seus efeitos colaterais.

	Medicamentos	*Efeitos Colaterais*[33,34,35,36]
Neurolépticos Típicos ou de Primeira Geração	Clorpromazina Perfenazina Trifluoperazina Tiotixeno Haloperidol	- Distúrbio Motor (Tremor, discinesia tardia, distonia, acatisia, coreoatetose) - Anedonia (ausência de prazer) - Ganho moderado de peso - Sedação - Temperatura desregulada - Hiperprolactinemia - Ginecomastia - Hipotensão - Fotosensibilidade cutânea - Risco de arritmia fatal
Neurolépticos Atípicos ou de Segunda Geração	Clozapina Risperidona Olanzapina Quetiapina Ziprazidona Aripiprazola Amisulfoprida	- Agranulocitose (clozapina) - Ganho de peso - *Diabetes mellitus* - Hipercolesteromia - Opacidade Visual - Hipotensão - Sedação - Miocardite - Disturbio motor leve

1.1.2 Genética e Esquizofrenia

Como já descrito anteriormente, a SCZ é resultado de uma interação de fatores exógenos e endógenos. As variáveis endógenas atuantes para o estabelecimento da SCZ certamente não são fatores isolados e sim uma interconexão de uma série de fatores como, por exemplo, mediadores

[33] Satanove A, McIntosh JS. Phototoxic reactions induced by high doses of chlorpromazine and thiorizadine. JAMA 1967;200:209-212.
[34] Cox B, Lee TF. Do central dopamine receptors have a physiological role in termorugulation? Br J Pharmacol 1977; 61:83-86.
[35] Bressan RA, Costa DC, Jones HM, Ell PJ, Pilowsky LS. Typical antipsychotic; drugs – D(2) receptor occupancy and depressive symptoms in schizophrenia. Schizophr Res 2002;56:31-36.
[36] Smith S, Wheeler MJ, Murray R, O'Keane V. The effects of antipsychotics-induced hyperprolactinaemia on the hypothalamic-pituitary-gonadal axis. J. Clin Psychopharmacol 2002; 22:109-114.

neuroquímicos associados a disfunções no neurodesenvolvimento. E cada um destes fatores, que provavelmente envolvem uma pré-disposição genética para que se manifestem, certamente contém subfatores que se complementam.

Pesquisas já comprovaram esta forte predisposição genética como uma das causas da SCZ. A freqüência mundial e a similaridade evidente de sintomas e do curso da doença nas diversas populações têm mostrado que a SCZ não tem aspecto ambiental ou étnico que pareça alterar a prevalência da doença em certas regiões ou em populações específicas. No entanto, a suscetibilidade à SCZ está claramente relacionada a fatores genéticos; estudos familiares, de gêmeos e indivíduos adotados mostraram que o risco mórbido de SCZ nos familiares está relacionado com o grau de genes compartilhados. Os estudos de gêmeos foram fundamentais no estabelecimento de uma contribuição genética importante na etiologia da SCZ. Eles determinaram que a taxa de concordância para SCZ era da ordem de 41–65% entre gêmeos monozigóticos, e de 0–28% entre gêmeos dizigóticos e outros irmãos, e a herdabilidade foi estimada em aproximadamente 80–85% [revisado em 37]. Além disso, é conhecido que o risco de desenvolver a doença para os demais membros da família é diretamente proporcional ao grau de parentesco com o indivíduo afetado (Figura 1). E ainda, os estudos de adoção sugerem fortemente que esses riscos ocorrem devido a fatores genéticos e não estritamente ambientais[38].

Análises de alterações cromossômicas e estudos de associação[39] fornecem claras evidências sobre a variável genética atuante na SCZ que, segundo dados genético-epidemiológicos, é um modelo de herança poligênica, no qual diversos genes de pequeno efeito contribuem para um aumento no risco e susceptibilidade à doença[40,41,42].

[37] Cardno AG, Gottesman II. Twin studies of schizophrenia: from bow-and-arrow concordances to star wars Mx and functional genomics. Am J Med Genet 2000 97: 12-7.
[38] Kendler KS, Gruenberg AM. An independent analysis of the Danish Adoption Study of Schizophrenia. VI. The relationship between psychiatric disorders as defined by DSM-III in the relatives and adoptees. Arch Gen Psychiatry 1984 41: 555-64.
[39] Baron M. Genetics of schizophrenia and the new millennium: progress and pitfalls. Am J Hum Genet. 2001; 68(2): 299-312.
[40] McGue M, Gottesman, II. 1989. A single dominant gene still cannot account for the transmission of schizophrenia. Arch Gen Psychiatry 46:478-480
[41] Cannon TD, van Erp TG, Glahn DC. 2002. Elucidating continuities and discontinuities between schizotypy and schizophrenia in the nervous system. Schizophr Res 54:151-156.
[42] Owen MJ. 2005. Genomic approaches to schizophrenia. Clin Ther 27 Suppl A:S2-7.

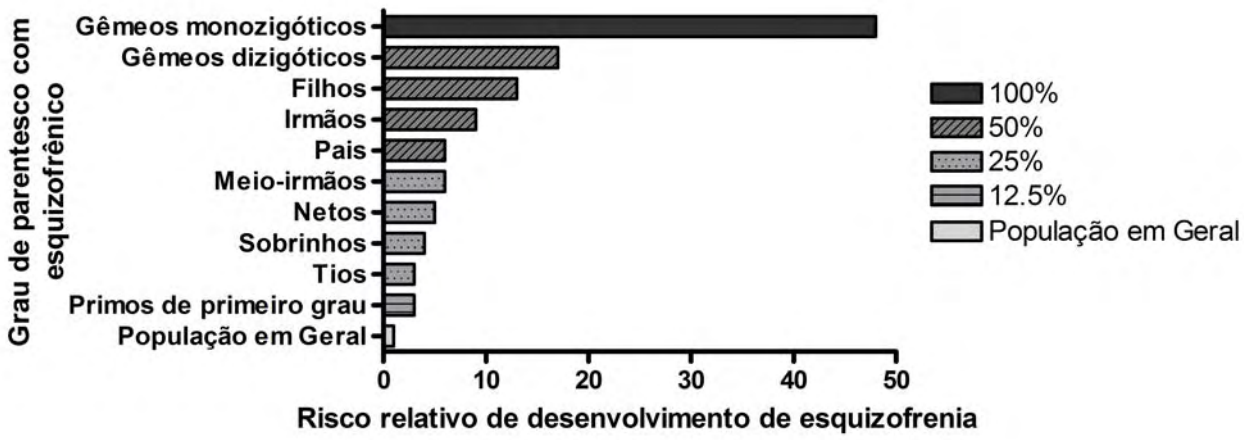

Figura 1: Risco de desenvolvimento de SCZ para parentes de indivíduos com SCZ, conforme Purim 2006[43], adapatado de Gottesman[44]. A legenda se refere ao grau de compartilhamento genético.

1.1.3 Neuroquímica da Esquizofrenia

Alterações neuroquímicas consistentemente observadas em tecidos humanos, derivados de pacientes portadores da SCZ deram origem às teorias (que podem ser vistas como complementares) que explicam as prováveis causas desta complexa neuropatologia.

1.1.3.1 Teoria Dopaminérgica

A hipótese dopaminérgica propõe que os sintomas psicóticos observados em pacientes com SCZ são devidos a uma hiperatividade dopaminérgica, resultado da quantidade aumentada de dopamina nas fendas sinápticas ou ainda devido ao aumento da quantidade de receptores de dopamina o que sensibilizaria a ação deste neurotransmissor [revisado em 45] . Esta hipótese surgiu com muita força devido às seguintes descobertas: a) os primeiros antipsicóticos usados no tratamento da SCZ eram antagonistas dos receptores de dopamina do tipo D2[46]; b) algumas drogas que mimetizam os efeitos psicóticos da SCZ (tais como a anfetamina e a cocaína), são agonistas dopaminérgicos indiretos, já que aumentam a transmissão

[43] Purim, SP. Neurogênese e esquizofrenia: estudo molecular de associação. Tese do Instituto de Química, Depto de Bioquímica - Universidade de São Paulo. Disponível em http://www.teses.usp.br/teses/disponiveis/46/46131/tde-12022007-150206/
[44] Gottesman I. 1991. Schizophrenia genesis: The origins of madness. New York: W-H Freeman.
[45] Harrison PJ. The neuropathology of schizophrenia. Brain 1999, 122, 593-624.
[46] Peroutka SJ, Snyder SH.Long-term antidepressant treatment decreases spiroperidol-labeled serotonin receptor binding.Science. 1980 Oct 3;210(4465):88-90.

dopaminérigica[47]; c) devido à descoberta de que os receptores dopaminérgicos, principalmente do tipo D2, estão aumentados em cérebros de pacientes com SCZ, apesar de também já ter havido relatos de alterações de receptores dos tipos D1 e D3[48,49].

Apesar da longevidade e da solidez dos achados que suportam esta hipótese, a mesma não consegue explicar a SCZ em sua totalidade pois: a) o bloqueio da neurotransmissão dopaminérgica não alivia totalmente os sintomas da SCZ, e sua ação se limita aos seus sintomas positivos[50]; b) embora os sintomas positivos sejam reduzidos pelo bloqueio da transmissão dopaminérgica, os níveis de metabólitos e de receptores de dopamina medidos antes e após o tratamento, permanecem dentro da variação de valores normais, dados que geram certa dificuldade de interpretação[51,52]; c) o papel da dopamina no cérebro é mais complexo do que a ação de um simples "interruptor" para sintomas psicóticos[53]; d) uma série de estudos de associação feitos com genes codificadores dos receptores de dopamina são pouco reprodutíveis além de contraditórios[54,55].

1.1.3.2 Teoria Serotonérgica

A idéia da disfunção serotonérgica como uma das causas da SCZ surgiu nos anos 50 devido à descoberta de que o LSD (ácido D-lisérgico), que provoca sintomas psicóticos, é um agonista da serotonina[56]. Mais tarde o envolvimento

[47] Meltzer HY, Stahl SM. The dopamine hypothesis of schizophrenia: a review. Schizophr Bull. 1976;2(1):19-76.
[48] Okubo Y, Suhara T, Suzuki K, Kobayashi K, Inoue O, Terasaki O, Someya Y, Sassa T, Sudo Y, Matsushima E, Iyo M, Tateno Y, Toru M. Decreased prefrontal dopamine D1 receptors in schizophrenia revealed by PET. Nature. 1997 Feb 13;385(6617):634-636.
[49] Gurevich EV, Bordelon Y, Shapiro RM, Arnold SE, Gur RE, Joyce JN. Mesolimbic dopamine D3 receptors and use of antipsychotics in patients with schizophrenia. A postmortem study. Arch Gen Psychiatry 1997; 54:225-232.
[50] Callicott JH, Egan MF, Mattay VS, Bertolino A, Bone AD, Verchinksi B, Weinberger DR. Abnormal fMRI response of the dorsolateral prefrontal cortex in cognitively intact siblings of patients with schizophrenia. Am J Psychiatry 2003 160: 709-19.
[51] Farde L, Hall H, Ehrin E, Sedvall G. Quantitative analysis of D2 dopamine receptor binding in the living human brain by PET. Science 1986 231: 258-61.
[52] Pickar D, Labarca R, Doran AR, Wolkowitz OM, Roy A, Breier A, Linnoila M, Paul SM. Longitudinal measurement of plasma homovanillic acid levels in schizophrenic patients: correlation with psychosis and response to neuroleptic treatment. Arch Gen Psychiatry 1986 43: 669-76.
[53] Freedman R. Schizophrenia. N Engl J Med 2003 349: 1738-49.
[54] Kohn Y, Ebstein RP, Heresco-Levy U, Shapira B, Nemanov L, Gritsenko I, Avnon M, Lerer B. Dopamine D4 receptor gene polymorphisms: relation to ethnicity, no association with schizophrenia and response to clozapine in Israeli subjects. Eur Neuropsychopharmacol 1997 7:39-43.
[55] Mihara K, Suzuki A, Kondo T, Nagashima U, Ono S, Otani K, Kaneko S. No relationship between Taq1 a polymorphism of dopamine D(2) receptor gene and extrapyramidal adverse effects of selective dopamine D(2) antagonists, bromperidol, and nemonapride in schizophrenia: a preliminary study. Am J Med Genet 2000 96:422-424.
[56] Shaw E, Woolley DW. Some serotonin-like activities of LSD. Science 1956 124:121-123.

dos receptores 5-HT2A foi explicado pela ação dos mesmos como potencializadores dos efeitos serotonérgicos [revisado em 57].

Estudos que comprovam o papel da serotonina na SCZ revelaram a expressão diminuída dos receptores 2A de serotonina no córtex frontal de cérebros de pacientes com SCZ[58] e a expressão cortical aumentada de receptores 1A[59]. Alterações nos níveis de receptores 1A e 2A foram observadas em pacientes não medicados, sugerindo o papel do sistema serotonérgico no desenvolvimento da doença[60].

Kapur e Remington[61] relataram a interação da serotonina com a dopamina e Harrison relatou que os baixos níveis do principal metabólito da serotonina (o ácido 5-hidroxiindolacético - 5-HIAA) no liquido encefalorradiquiano pode estar relacionado com os sintomas negativos da SCZ [53].

1.1.3.3 Teoria Glutamatérgica

Antagonistas dos receptores de glutamato do subtipo NMDA produzem psicoses parecidas com as apresentadas por pacientes com SCZ[62]. Outras evidências surgiram mais tarde em estudos de tecido cerebral de pacientes com SCZ, como a expressão diminuída de receptores não-NMDA de glutamato, aumento cortical de algumas subunidades dos receptores NMDA, diminuição da liberação de glutamato cortical e concentrações alteradas de glutamato e seus metabólitos [revisado em 53]. Além disso, é descrita a conexão entre os sistemas glutamatérgico e dopaminérgico[63] bem como glutamatérgico com serotonérgico[57].

[57] Aghajanian GK, Marek GJ. Serotonin model of schizophrenia: emerging role of glutamate mechanisms. Brain Reser. Rev. 2000 31: 302-312.
[58] Harrison PJ. Neurochemical alterations in schizophrenia affecting the putative targets of atypical antipsychotics: focus on dopamine (D1, D3, D4) and 5-HT2A receptors. Br J Psychiatry 1999; 174 Suppl 38: 41–51
[59] Burnet PW, Eastwood SL, Harrison PJ. [3H]WAY-100635 for 5-HT1A receptor autoradiography in human brain: a comparison with [3H]8-OH-DPAT and demonstration of increased binding in the frontal cortex in schizophrenia. Neurochem Int 1997; 30: 565–74
[60] Trichard C, Paillère-Martinot ML, Attar-Levy D, Blin J, Feline A, Martinot JL. No serotonin 5-HT2A receptor density abnormality in the cortex of schizophrenic patients studied with PET. Schizophr Res 1998; 31: 13–7.
[61] Kapur S, Remington G. Serotonin-dopamine interaction and its relevance to schizophrenia. Am J Psychiatry 1996; 153: 466–76.
[62] Javitt DC, Zukin SR. Recent advances in the phencyclidine model of schizophrenia. Am. J Psychiatry 1991 148: 1301-1308.
[63] Carlson M, Carlson A. Schizophrenia: a subcortical neurotransmitter imbalance síndrome? Schizophr. Bull 1990; 16:425-432.

1.1.3.4 Teoria Sináptica

Desde o início dos estudos em busca da compreensão da SCZ sabe-se que há um estado alterado das funções sinápticas, bem como sua plasticidade[64]. As hipóteses acima descritas são algumas das maiores provas desta disfunção. O defeito sináptico em cérebros de pacientes com SCZ pode levar ao déficit de memória, mau funcionamento do circuito hipocampal e má formação das sinapses[65]. Além disso, uma redução na neurotransmissão[66], além de padrões anormais de ativação e alterações na transmissão sináptica já foram demonstrados em cérebros de pacientes com SCZ[67]. Potenciais marcadores moleculares revelados em estudos de expressão gênica em cérebros de pacientes com SCZ que comprovam a disfunção sináptica estão citados na Tabela 3. Estas evidências sugerem que alterações na transmissão sináptica e conectividade neuronal estejam entre os principais fatores que causam a SCZ[68].

1.1.4 A Esquizofrenia Como Uma Doença de Neurodesenvolvimento

Várias evidências têm dado suporte a uma das hipóteses mais aceitas atualmente, a qual sugere que alterações no neurodesenvolvimento contribuem para a suscetibilidade à SCZ[69,70,71]. Primeiramente, estudos clínicos mostraram que pacientes com SCZ manifestam pequenas alterações de comportamento na infância mesmo antes do início da doença. Segundo, avanços recentes nas técnicas de imagem, tais como a ressonância magnética, fornecem evidências concretas de que pequenas alterações ocorrem durante o desenvolvimento do sistema nervoso central de pacientes com SCZ. Tais anormalidades incluem aumentos consistentes no tamanho ventricular no início da SCZ, com alterações notáveis em algumas áreas, incluindo córtex pré-frontal e hipocampo. Gilmore *et*

[64] Owen MJ, O'Donovan MC, Harrison PJ. Schizophrenia: a genetic disorder of the synapse? BMJ, January 22, 2005; 330(7484): 158 – 159.
[65] Ben-Shachar D, Laifenfeld D. Mitochondria, synaptic plasticity, and schizophrenia. Int Rev Neurobiol. 2004;59:273-96.
[66] Selemon LD, Goldman-Rakic PS. 1999. The reduced neuropil hypothesis: a circuit based model of schizophrenia. Biol Psychiatry 45:17-25.
[67] Kegeles LS, Humaran TJ, Mann JJ. 1998. In vivo neurochemistry of the brain in schizophrenia as revealed by magnetic resonance spectroscopy. Biol Psychiatry 44:382-398.
[68] Frankle WG, Lerma J, Laruelle M. 2003. The synaptic hypothesis of schizophrenia. Neuron 39:205-216.
[69] Weinberger DR, Torrey EF, Neophytides AN, Wyatt RJ. Lateral cerebral ventricular enlargement in chronic schizophrenia. Arch Gen Psychiatry 1979 36: 735-739.
[70] Weinberger DR. Implications of normal brain development for the pathogenesis of schizophrenia. Arch Gen Psychiatry 1987 44: 660-669.
[71] Sawa A, Snyder SH Schizophrenia: Diverse Approaches to a Complex Disease. Science; 2002 296; 692-695.

al.[72] detectaram déficit no desenvolvimento cerebral em fetos e recém-nascidos de mulheres com SCZ. Terceiro, alguns estudos de neuropatologia indicam que não ocorre perda total no número de neurônios, mas sim redução no tamanho dessas células. As alterações na cito-arquitetura incluem variabilidade da orientação celular e diminuição das estruturas sinápticas.

Alguns autores consideram a SCZ como uma doença de neurodesenvolvimento e não como uma doença neurodegenerativa (caracterizada pela proliferação das células da glia e degeneração dos neurônios). Pacientes com SCZ não apresentam proliferação glial[73], entretanto apresentam morte neuronal pela sinalização dos sistemas de neurotransmissores, que geram mudanças morfológicas cerebrais progressivas[74]. Sendo assim, alguns autores consideram a SCZ uma doença de neurodesenvolvimento com um importante componente de neurodegeneração.

1.1.5 Alterações Neuroanatômicas em Pacientes com Esquizofrenia

Em cérebros de pacientes com SCZ foram observadas diminuições significativas no número de interneurônios e uma diminuição da migração dos neurônios intersticiais para o córtex[75,76], bem como um decréscimo no número total de neurônios em algumas áreas do cérebro[77].

Em um achado consistente, Kubicki *et al.*[78] mostraram através de imagem por ressonância magnética (MRI), o alargamento dos ventrículos e a diminuição do volume de muitas regiões cerebrais, incluindo o hipocampo e o córtex temporal superior. Apesar dessa aparente diminuição do tecido cerebral, a MRI funcional (fMRI) revela hiperatividade no hipocampo e no córtex pré-frontal dorso-lateral, o que talvez esteja consistente com a perda da função neuronal inibitória[79,80].

[72] Gilmore JH, van Tol J, Kliewer MA, Silva SG, Cohen SB, Hertzberg BS, Chescheir NC. Mild ventriculomegaly detected in utero with ultrasound: clinical associations and implications for schizophrenia. Schizophr Res 1998 33: 133-40.
[73] Sawa A, Kamiya A. Elucidating the pathogenesis of schizophrenia. BMJ 2003 327: 632-33.
[74] Pérez-Neri I, Ramírez-Bermúdez J, Montes S, Ríos C. Possible mechanisms of neurodegeneration in schizophrenia. Neurochem Res. 2006 Oct;31(10):1279-94.
[75] Akbarian S, Kim JJ, Potkin SG, Hetrick WP, Bunney WE Jr, Jones EG. Maldistribution of interstitial neurons in prefrontal white matter of the brains of schizophrenic patients. Arch Gen Psychiatry 1996 53: 425-36.
[76] Benes FM, Kwok EW, Vincent SL, Todtenkopf MS. A reduction of nonpyramidal cells in sector CA2 of schizophrenics and manic depressives. Biol Psychiatry 1998 44: 88-97.
[77] Jeste DV, Lohr JB. Hippocampal pathologic findings in schizophrenia: a morphometric study. Arch Gen Psychiatry 1989 46: 1019-24.
[78] Kubicki M, Shenton ME, Salisbury DF, Hirayasu Y, Kasai K, Kikinis R, Jolesz FA, McCarley RW. Voxel-based morphometric analysis of gray matter in first episode schizophrenia. Neuroimage 2002 17: 1711-9.
[79] Tamminga CA, Thaker GK, Buchanan R, Kirkpatrick B, Alphs LD, Chase TN, Carpenter WT.Limbic system abnormalities identified in schizophrenia using positron emission tomography with fluorodeoxyglucose and neocortical alterations with deficit syndrome. Arch Gen Psychiatry. 1992 Jul;49(7):522-30.

Utilizando fMRI, Callicott *et al*.[81] encontraram evidências diretas de uma alteração fisiológica primária na função do córtex pré-frontal dorso-lateral em indivíduos com elevado risco desenvolver SCZ, mesmo na ausência de qualquer manifestação cognitiva anormal.

1.1.6 Principais Marcadores Moleculares na Esquizofrenia

Os estudos de expressão global de genes e proteínas de tecidos de pacientes SCZ é relativamente recente e teve grande ascensão do início desta década até os dias atuais com muitos estudos de transcriptoma utilizando *microarrays*. Os dados de proteoma obtidos, apesar da pequena quantidade de trabalhos publicados, confirmam parte significativa dos dados obtidos pelo transcriptoma, mostrando que genes envolvidos com o metabolismo de oligodendrócitos, com o metabolismo energético e com o metabolismo sináptico tem importante papel na SCZ.

1.1.6.1 Marcadores de RNA

Os marcadores de RNA mais consistentes, observados a partir do estudo de tecido cerebral de pacientes portadores da SCZ consistem em genes que codificam para proteínas relacionadas ao metabolismo de oligodendrócitos e ao metabolismo sináptico (descrito no item 1.1.3.4 desta tese). Observaram-se também uma diferença de expressão em genes relacionados ao metabolismo energético, dados fortemente confirmados por análises proteômicas utilizando tecido de pacientes com SCZ com consistentes achados.

Oligodendrócitos são células da neuroglia, responsáveis pela mielinização dos axônios neuronais do sistema nervoso central (SNC). Estudos de RNA em busca de potenciais biomarcadores para a SCZ revelaram a alteração de diversos genes relacionados com o metabolismo de oligodendrócitos, sugerindo o importante papel deste tipo celular na patogenia da SCZ, inclusive desviando a visão centrada na disfunção dopaminérgica da SCZ. Alguns destes marcadores de RNA

[80] Cecil KM, Lenkinski RE, Gur RE, Gur RC. Proton magnetic resonance spectroscopy in the frontal and temporal lobes of neuroleptic naïve patients with schizophrenia. Neuropsychopharmacology 1999 20: 131-40.
[81] Callicott JH, Egan MF, Mattay VS, Bertolino A, Bone AD, Verchinksi B, Weinberger DR. Abnormal fMRI response of the dorsolateral prefrontal cortex in cognitively intact siblings of patients with schizophrenia. Am J Psychiatry 2003 160: 709-19.

foram confirmados por estudos de proteoma de tecidos de pacientes com SCZ como descrito na Tabela 3.

Uma revisão detalhada dos potenciais marcadores moleculares de RNA é apresentada no Capítulo 2 desta tese.

1.1.6.2 Marcadores Protéicos

Os marcadores protéicos mais consistentes, observados a partir de estudos com amostras cerebrais de pacientes com SCZ consistem em proteínas relacionadas ao metabolismo energético celular, confirmando dados prévios obtidos em estudos de transcriptoma[82], apesar de alguns estudos de proteoma confirmarem robustamente também a expressão diferencial de genes relacionados ao metabolismo de oligodendrócitos. A alteração do metabolismo energético na SCZ já foi extensamente descrita[83,84,85,86] bem como sua relação com a plasticidade neuronal e função sináptica[87], além das evidencias de estresse oxidativo em cérebros de pacientes com SCZ[88,89]. Estes dados, sendo alguns apresentados na Tabela 3, suportam a hipótese de alguns pesquisadores que se referem à SCZ como uma "doença mitocondrial".

[82] Middleton FA, Mirnics K, Pierri JN, Lewis DA, Levitt P. Gene expression profiling reveals alterations of specific metabolic pathways in schizophrenia. J Neurosci. 2002 Apr 1;22(7):2718-29.
[83] Ben-Shachar D. Mitochondrial dysfunction in schizophrenia: a possible linkage to dopamine. J Neurochem. 2002 Dec;83(6):1241-51.
[84] Bubber P, Tang J, Haroutunian V, Xu H, Davis KL, Blass JP, Gibson GE. Mitochondrial enzymes in schizophrenia. J Mol Neurosci. 2004; 24(2):315-321.
[85] Karry R, Klein E, Ben Shachar D. Mitochondrial complex I subunits expression is altered in schizophrenia: a postmortem study. Biol Psychiatry. 2004; 55(7):676-684.
[86] Martorell L, Segues T, Folch G, Valero J, Joven J, Labad A, Vilella E. New variants in the mitochondrial genomes of schizophrenic patients. Eur J Hum Genet. 2006;14(5):520-528.
[87] Ben-Shachar D, Laifenfeld D. Mitochondria, synaptic plasticity, and schizophrenia. Int Rev Neurobiol. 2004;59:273-96.
[88] Yao JK, Reddy RD, van Kammen DP. Oxidative damage and schizophrenia: an overview of the evidence and its therapeutic implications. CNS Drugs. 2001;15(4):287-310.
[89] Prabakaran S, Swatton JE, Ryan MM, Huffaker SJ, Huang JT, Griffin JL, Wayland M, Freeman T, Dudbridge F, Lilley KS, Karp NA, Hester S, Tkachev D, Mimmack ML, Yolken RH, Webster MJ, Torrey EF, Bahn S. Mitochondrial dysfunction in schizophrenia: evidence for compromised brain metabolism and oxidative stress. Mol Psychiatry. 2004 Jul;9(7):684-97, 643.

*Tabela 3: Principais classes de genes e proteínas e seus representantes previamente descritos em estudos de transcriptoma e proteoma. Biomaracadores indicados por * foram encontrados em trabalhos de transcriptoma e proteoma. Os indicados por - foram encontrados em trabalhos de transcriptoma e os indicados por = foram encontrados em trabalhos de proteoma.*

Via Bioquímica	*Potenciais Biomarcadores*	*Autores* *(=trabalhos de proteômica)* *(-trabalhos de transcriptoma)*
Metabolismo de Oligodendrócitos	* CNP – fosfodiesterase 3'de nucleotídio ciclico 2' 3' * MOG - Proteína Oligodendroglial de Mielina * PLP - Proteína Proteolipídica * GSN – Gelsolina * MAG - Glicoproteína Associada à Mielina * TF – Transferrina - ERBB3 - Oncogene v-erb-b2 - MAL - Proteína Associada à Maturação de Linfócito T; Proteína de Mielina e Linfócito. - CLDN11 - Claudina 11; Proteína Específica de Oligodendrócito. - PLLP - Plasmolipina - QKI – Proteína "Quaking"	- Hakak et al., 2001[90] - Tkachev et al., 2003[91] - Aston et al., 2004[92] = Prabakaran et al., 2004[93] - Katsel et al., 2005[94] - Dracheva et al., 2006[95] - McCullumsmith et al., 2007[96] - Arion et al., 2007[97]
Metabolismo Energético	* ALDOC - Aldolase C * GOT2 - Transaminase Glutâmica-Oxalacética 2 * MDH1 - Malato Desidrogenase 1 * ATP5A1 - F1 do Complexo Alfa da ATP Sintase Mitocondrial * OAT - Ornitina Aminotransferase * OXCT1 - 3-Oxoacido CoA Transferase - Azin1 - Inibidor de Antienzima - CRYM - Cristalina - TIMM17A - Translocase 17 de Membrana Mitocondrial Interna - USP14 - Protease 14 Ubiquitino-Específica - UCHL1 - Estearase L1 = CA2 - Anidrase Carbônica 2 = PKM1 - Piruvato Quinase = ACADS and ACADL - Acyl-CoA Oxidase Peroxisomal	- Middleton et al., 2002[98] = Prabakaran et al., 2004[93] = Clark et al., 2006[99] = Beasley et al., 2006[100]

[90] Hakak Y, Walker JR, Li C, Wong WH, Davis KL, Buxbaum JD, Haroutunian V, Fienberg AA. Genome-wide expression analysis reveals dysregulation of myelination-related genes in chronic schizophrenia. Proc Natl Acad Sci U S A. 2001 Apr 10;98(8):4746-51.
[91] Tkachev D, Mimmack ML, Ryan MM, Wayland M, Freeman T, Jones PB, Starkey M, Webster MJ, Yolken RH, Bahn S. Oligodendrocyte dysfunction in schizophrenia and bipolar disorder. Lancet. 2003 Sep 6;362(9386):798-805.
[92] Aston C, Jiang L, Sokolov BP. Microarray analysis of postmortem temporal cortex from patients with schizophrenia. J Neurosci Res. 2004 Sep 15;77(6):858-66.
[93] Prabakaran S, Swatton JE, Ryan MM, Huffaker SJ, Huang JT, Griffin JL, Wayland M, Freeman T, Dudbridge F, Lilley KS, Karp NA, Hester S, Tkachev D, Mimmack ML, Yolken RH, Webster MJ, Torrey EF, Bahn S. Mitochondrial dysfunction in schizophrenia: evidence for compromised brain metabolism and oxidative stress. Mol Psychiatry. 2004 Jul;9(7):684-97, 643.
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[95] Dracheva S, Davis KL, Chin B, Woo DA, Schmeidler J, Haroutunian V. Myelin-associated mRNA and protein expression deficits in the anterior cingulate cortex and hippocampus in elderly schizophrenia patients. Neurobiol Dis. 2006 Mar;21(3):531-40.
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[97] Arion D, Unger T, Lewis DA, Levitt P, Mirnics K. Molecular evidence for increased expression of genes related to immune and chaperone function in the prefrontal cortex in schizophrenia. Biol Psychiatry. 2007 Oct 1;62(7):711-21.
[98] Middleton FA, Mirnics K, Pierri JN, Lewis DA, Levitt P. Gene expression profiling reveals alterations of specific metabolic pathways in schizophrenia. J Neurosci. 2002 Apr 1;22(7):2718-29.
[99] Clark D, Dedova I, Cordwell S, Matsumoto I. A proteome analysis of the anterior cingulate cortex gray matter in schizophrenia. Mol Psychiatry. 2006; 11:459-470.
[100] Beasley CL, Pennington K, Behan A, Wait R, Dunn MJ, Cotter D. Proteomic analysis of the anterior cingulate cortex in the major psychiatric disorders: Evidence for disease-associated changes. Proteomics. 2006; 6(11):3414-3425.

Metabolismo Energético	de Cadeias Curta e Longa = ACAT2 - Acetil-Coenzima A Aciltransferase 2 = AGPS - Citrato Liase = CPT1, CPT2 - Carnitina Palmitoiltransferases 1 e 2 = ACO – Aconitase = ENO- Enolase = PDH - Piruvato Desidrogenase = GAPDH - gliceraldeído 3 fosfato desidrogenase	
Sinapse e plasticiadade neural	* YWHAH - Peptídeo ETA da proteína de ativação da Tirosina 3-Monooxigenase / Triptofano 5-Monooxigenase * CALM - Calmodulina - MARCKS – Substrato C de Quinase Rico em Alanina Miristoilado - GAP-43 - Proteína Associada ao Crescimento 43 - SCG-10 - Proteína Associada ao Crescimento Neuronal - SERPINI1 – Neuroserpina - NSF - Fator sensível a N-etil maleimido - SYN2 - Sinapsina II - RAB3C - Proteína de ligação ao GTP - AMPA2 - Receptor Ionotrópico de Glutamato	- Hakak et al., 2001[90] - Vawter et al., 2001[101] - Mirnics et al., 2000[102] - Aston et al., 2004[92] = Sivagnanasundaram et al., 2007[103]

1.1.6.3 Marcadores Moleculares no Sangue

Sendo a SCZ uma doença sem marcadores moleculares estabelecidos, pensou-se que se as alterações de expressão gênica ou protéica observadas no tecido cerebral provindo de pacientes com SCZ pudessem ser também detectadas nos biofluidos humanos, principalmente no sangue por ser um método menos invasivo de análise, o que permitiria o acompanhamento da doença através de marcadores moleculares para esta patogenia. Na Tabela 4 apresentamos os potenciais biomarcadores encontrados no tecido cerebral e confirmados em sangue de pacientes com SCZ. Além destes, estão relatados também alguns potenciais biomarcadores encontrados exclusivamente no sangue.

[101] Vawter MP, Barrett T, Cheadle C, Sokolov BP, Wood WH 3rd, Donovan DM, Webster M, Freed WJ, Becker KG. Application of cDNA microarrays to examine gene expression differences in schizophrenia. Brain Res Bull. 2001 Jul 15;55(5):641-50.
[102] Mirnics K, Middleton FA, Marquez A, Lewis DA, Levitt P. Molecular characterization of schizophrenia viewed by microarray analysis of gene expression in prefrontal cortex. Neuron. 2000 Oct;28(1):53-67.
[103] Sivagnanasundaram S, Crossett B, Dedova I, Cordwell S, Matsumoto I. Abnormal pathways in the genu of the corpus callosum in schizophrenia pathogenesis: a proteome study. Proteomics clin. appl. 2007: 1(10): 1291-1305.

Tabela 4: Potenciais biomarcadores encontrados no sangue de pacientes com esquizofrenia previamente encontrados ou não em tecido cerebral.

Autores que encontraram potenciais biomarcadores no sangue	*Gene com regulação alterada no sangue*
Vawter et al. (2004)[104]	- Neuropeptideo Y (NPY) (Encontrado com regulação alterada no cérebro por Hakak et al., 2001[90]) - Malato Desidrogenase (MDH1) (Encontrado com regulação alterada no cérebro por Hakak et al., 2001[90]; Middleton et al., 2002[99])
Tsuang et al. (2005)[105]	- Forbolina (APOBEC3B) - Adenilosucinato Sintetase (ADSS) - Proteína (ATM) - Galactina 10 (CLC) - Fator de Transcrição Associado a Morte Celular 1 (DIDO1) - Motif de Ligação à Quimiocina 1 (CXCL1) - Proteína S100 de ligação ao cálcio A9 (S100A9)
Glatt et al. (2005)[106]	- Gene de Translocação de Célula B, Antiproliferativa (BTG1) (com expressão aumentada no córtex pré-frontal dorsolateral (DLPFC) e com expressão diminuída no sangue) - Glicogênio Sintase Quinase 3 alfa (GSK3A) (com expressão diminuída no DLPFC e com expressão aumentada no sangue) - Ribonucleoproteina Heterogênia Nuclear A3 (HNRPA3) (com expressão aumentada no DLPFC e com expressão diminuída no sangue) - MHC de classe II, DR Beta 1 (HLA-DLB1) (Com expressão diminuída em DLPFC e no sangue)

[104] Vawter MP, Ferran E, Galke B, Cooper K, Bunney WE, Byerley W. Microarray screening of lymphocyte gene expression differences in a multiplex schizophrenia pedigree. Schizophr Res. 2004 Mar 1;67(1):41-52.
[105] Tsuang MT, Nossova N, Yager T, Tsuang MM, Guo SC, Shyu KG, Glatt SJ, Liew CC. Assessing the validity of blood-based gene expression profiles for the classification of schizophrenia and bipolar disorder: a preliminary report. Am J Med Genet B Neuropsychiatr Genet. 2005 Feb 5;133(1):1-5.
[106] Glatt SJ, Everall IP, Kremen WS, Corbeil J, Sásik R, Khanlou N, Han M, Liew CC, Tsuang MT. Comparative gene expression analysis of blood and brain provides concurrent validation of SELENBP1 up-regulation in schizophrenia. Proc Natl Acad Sci U S A. 2005 Oct 25;102(43):15533-8.

Glatt et al. (2005)[106]	- Proteína de ligação ao Selênio 1 (SELENBP1) (Com expressão aumentada em DLPFC e no sangue) - Fator de *Splicing* Rico em Arginina/Serina 1 (SFRS1) (com expressão aumentada no DLPFC e com expressão diminuída no sangue)
Middleton et al. (2005)[107]	- Alfa Catenina (CTNNA1) - Neuregulina 1 (NRG1) - Similar ao Fator de Transcrição 4 (TCFL4) - Herstatina (ERBB2) - Fator Derivado de Neurônio Moto-Sensorial (SMDF) - Citocromo P450 Família 1, Subfamilia B, Polipeptideo 1 (CYP1B1) - Proteína Quaking (QKI) - Fosfatase Duplo Específica 6 (DUSP6)
Bowden et al. (2006)[108]	- Gene de Diferenciação Endotelial 2 (Edg-2) - Fosfoproteína Ezrina-radixina-moesina 50 (EBP50) - Proteina Zinc-Finger associada a Myc (MAZ) - Receptor de Fator de Necrose Tumoral 2 (TNFR2)
Perl et al. (2006)[109]	- Receptor Nicotínico de Acetilcolina Alfa 7 (CHRNA7)
Mehler-Wex et al. (2006)[110]	- Subunidade 75-kDa do Complexo Mitocondrial I (NDUFS1)
Numata et al. (2007)[111]	- Domínio 5 de LIM e PDZ (PDLIM5)

[107] Middleton FA, Pato CN, Gentile KL, McGann L, Brown AM, Trauzzi M, Diab H, Morley CP, Medeiros H, Macedo A, Azevedo MH, Pato MT. Gene expression analysis of peripheral blood leukocytes from discordant sib-pairs with schizophrenia and bipolar disorder reveals points of convergence between genetic and functional genomic approaches. Am J Med Genet B Neuropsychiatr Genet. 2005 Jul 5;136(1):12-25.

[108] Bowden NA, Weidenhofer J, Scott RJ, Schall U, Todd J, Michie PT, Tooney PA. Preliminary investigation of gene expression profiles in peripheral blood lymphocytes in schizophrenia. Schizophr Res. 2006 Feb 28;82(2-3):175-83.

[109] Perl O, Strous RD, Dranikov A, Chen R, Fuchs S. Low levels of alpha7-nicotinic acetylcholine receptor mRNA on peripheral blood lymphocytes in schizophrenia and its association with illness severity. Neuropsychobiology. 2006;53(2):88-93.

[110] Mehler-Wex C, Duvigneau JC, Hartl RT, Ben-Shachar D, Warnke A, Gerlach M. Increased mRNA levels of the mitochondrial complex I 75-kDa subunit. A potential peripheral marker of early onset schizophrenia? Eur Child Adolesc Psychiatry. 2006 Dec;15(8):504-7.

[111] Numata S, Ueno S, Iga J, Yamauchi K, Hongwei S, Hashimoto R, Takeda M, Kunugi H, Itakura M, Ohmori T. Gene expression in the peripheral leukocytes and association analysis of PDLIM5 gene in schizophrenia. Neurosci Lett. 2007 Mar 19;415(1):28-33

1.1.7 Esquizofrenia: Visão Molecular Atual

A visão atual é de que a SCZ é provavelmente derivada de um acúmulo de pequenas alterações genômicas, somada à desregulação transcricional e traducional de vários genes e proteínas, e associada a importantes fatores ambientais. Individualmente, cada um destes fatores (genômicos, transcricionais, traducionais e ambientais) não são suficientes nem necessários para provocar o surgimento da doença. No entanto, a somatória destes fatores parece ter um papel fundamental para o seu estabelecimento. Determinar o painel de genes expressos (transcriptoma) e de proteínas expressas (proteoma) de tecidos de pacientes SCZ converge para a compreensão sistemática da doença, bem como no estabelecimento de potenciais alvos terapêuticos.

Os recentes desenvolvimentos em neuropatologia, técnicas de imagem cerebral, genética molecular, além de uma nova geração de medicamentos, têm possibilitado uma melhor compreensão da patofisiologia da SCZ e melhorado significativamente o seu tratamento. Contudo, esta ainda é uma doença enigmática, não totalmente elucidada, e que representa uma sobrecarga substancial aos pacientes, suas famílias e para a sociedade.

1.2 Análise Transcricional em Larga Escala

Após o término do sequenciamento do genoma humano, ficou evidente que apenas sequências genômicas completas não são suficientes para elucidar a complexidade de diversos processos biológicos, ou da maioria das doenças humanas[112]. Um exemplo representativo é a nossa incapacidade de compreender os processos que levam à diferenciação celular. Como um neurônio e, digamos, um hepatócito e uma célula muscular de um indivíduo, que têm o mesmo genoma, e apresentam tamanhos, funções e fisiologia tão distintas, fruto da expressão gênica e protéica diferencial nestas células? Questões deste tipo inauguraram a era Pós-Genômica, na busca do conhecimento biológico magno que permitisse melhor compreender os ricos dados genômicos.

[112] Pandey, A., Mann, M. Proteomics to study genes and genomes. Nature 2000, 405, 837-846.

O termo "transcriptoma" foi introduzido por Velculescu et al. em 1997[113] como o conjunto de genes expressos num determinado organismo, complementado mais tarde como o conjunto de genes expressos em determinado momento, numa determinada condição imposta pelo meio[114].

Os dados obtidos em estudos de transcriptoma podem revelar importantes informações sobre a regulação da expressão gênica, os fenótipos gerados e a compreensão sistemática da célula em questão. As tecnologias atualmente disponíveis permitem avaliar com precisão a qualidade e a quantidade dos diversos transcritos, tornando os estudos de transcriptoma muito ricos, completos e complexos.

Transcriptomas vêm sendo estudados por diversas abordagens, sendo que as principais serão descritas a seguir.

1.2.1 Etiquetas de Seqüências Expressas (Expressed Sequenced Tags - EST)

A idéia deste método pioneiro usado para determinação do perfil da expressão gênica foi do grupo de J. Craig Venter[115], um dos pioneiros dos estudos do genoma humano. Sucintamente, o RNA total ou mensageiro das amostras de interesse é extraído, transformado em cDNA dupla-fita, clonado em vetores de sequenciamento e sequenciado. Os insertos são sequenciados por uma leitura única a partir de uma ou de ambas extremidades, o que em geral leva ao sequenciamento parcial da molécula clonada. Deste modo, os genes expressos no tecido de interesse são revelados pela leitura de um fragmento de seu cDNA. As seqüências obtidas são relativamente longas (de 500 a 800 nucleotídeos) e permitem o seu mapeamento inequívoco no genoma. Os resultados podem gerar informações sobre novos genes, além da diversidade transcricional do tecido de interesse como mutações, *splicing* alternativo e polimorfismos. Quando o sequenciamento é feito a partir de bibliotecas de cDNA não normalizadas, os dados são geralmente quantitativos, e refletem a expressão do gene em questão.

[113] Velculescu VE, Zhang L, Zhou W, Vogelstein J, Basrai MA, Bassett DE Jr, Hieter P, Vogelstein B, Kinzler KW.Characterization of the yeast transcriptome.Cell. 1997 Jan 24;88(2):243-51.
[114] Tan SS, Gunnersen J, Job C.Global gene expression analysis of developing neocortex using SAGE.Int J Dev Biol. 2002;46(4):653-60.
[115] Adams MD, Dubnick M, Kerlavage AR, Moreno R, Kelley JM, Utterback TR, Nagle JW, Fields C, Venter JC. Sequence identification of 2,375 human brain genes. Nature. 1992 Feb 13;355(6361):632-4.

Uma das limitações da técnica é quanto ao custo do sequenciamento, visto que para a cobertura completa do transcriptoma, há a necessidade da geração centenas de milhares de seqüências. Protocolos alternativos na utilização da técnica de EST surgiram e vêm auxiliando a descoberta gênica em diversas situações[116,117].

Em neuropsiquiatria, o uso de ESTs tem sido aplicado em estudos sobre a Doença de Parkinson[118,119], epilepsia[120] e SCZ[121].

1.2.2 Microarranjos de cDNA (cDNA Microarrays)

O desenvolvimento dos microarranjos de cDNA possibilitou o surgimento de uma das ferramentas mais poderosas para investigação de expressão gênica em larga escala, por permitir uma análise rápida, simultânea e sensível de milhares de genes em um grande numero de amostras. Estudos voltados para o diagnóstico, prognóstico, progressão de doenças e resposta a medicamentos são alguns do que utilizaram esta tecnologia na pesquisa médica.

Basicamente desenvolvida para avaliação de expressão gênica, esta técnica hoje também possibilita a análises de mutações[122,123], *in situ* microarrays[124,125],

[116] Soares MB, Bonaldo MF, Jelene P, Su L, Lawton L, Efstratiadis A. Construction and characterization of a normalized cDNA library. Proc Natl Acad Sci U S A. 1994 Sep 27;91(20):9228-9232.
[117] Dias-Neto E, Correa RG, Verjovski-Almeida S, Briones MR, Nagai MA, da Silva W Jr, Zago MA, Bordin S, Costa FF, Goldman GH, Carvalho AF, Matsukuma A, Baia GS, Simpson DH, Brunstein A, de Oliveira PS, Bucher P, Jongeneel CV, O'Hare MJ, Soares F, Brentani RR, Reis LF, de Souza SJ, Simpson AJ. Shotgun sequencing of the human transcriptome with ORF expressed sequence tags. Proc Natl Acad Sci U S A. 2000 Mar 28;97(7):3491-6.
[118] Lu L, Neff F, Alvarez-Fischer D, Henze C, Xie Y, Oertel WH, Schlegel J, Hartmann A. Gene expression profiling of Lewy body-bearing neurons in Parkinson's disease. Exp Neurol. 2005 Sep;195(1):27-39.
[119] Kim JM, Lee KH, Jeon YJ, Oh JH, Jeong SY, Song IS, Kim JM, Lee DS, Kim NS. Identification of genes related to Parkinson's disease using expressed sequence tags. DNA Res. 2006 Dec 31;13(6):275-86.
[120] Avedissian M, Longo BM, Jaqueta CB, Schnabel B, Paiva PB, Mello LE, Briones MR. Hippocampal gene expression analysis using the ORESTES methodology shows that homer 1a mRNA is upregulated in the acute period of the pilocarpine epilepsy model. Hippocampus. 2007;17(2):130-6.
[121] Bell R, Munro J, Russ C, Powell JF, Bruinvels A, Kerwin RW, Collier DA. Systematic screening of the 14-3-3 eta (eta) chain gene for polymorphic variants and case-control analysis in schizophrenia. Am J Med Genet. 2000 Dec 4;96(6):736-43.
[122] Klevering BJ, Yzer S, Rohrschneider K, Zonneveld M, Allikmets R, van den Born LI, Maugeri A, Hoyng CB, Cremers FP. Microarray-based mutation analysis of the ABCA4 (ABCR) gene in autosomal recessive cone-rod dystrophy and retinitis pigmentosa.Eur J Hum Genet. 2004 Dec;12(12):1024-32.
[123] Van Bogaert P, Azizieh R, Désir J, Aeby A, De Meirleir L, Laes JF, Christiaens F, Abramowicz MJ. Mutation of a potassium channel-related gene in progressive myoclonic epilepsy. Ann Neurol. 2007 Jun;61(6):579-86.
[124] Kononen J, Bubendorf L, Kallioniemi A, Bärlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi OP. Tissue microarrays for high-throughput molecular profiling of tumor specimens.Nat Med. 1998 Jul;4(7):844-7.
[125] Simon R, Mirlacher M, Sauter G. Tissue microarrays.Biotechniques. 2004 Jan;36(1):98-105.

sequenciamento de genes[126,127] e análise de eventos de eventos de *splicing* alternativo[128,129].

A idéia dos microarranjos de cDNA surgiu a partir do conhecimento dos conceitos da dupla-hélice do DNA (por Watson e Crick em 1953) e da descoberta de que a dupla-fita de DNA poderia ser separada por tratamento alcalino ou por calor permitindo a posterior hibridização do DNA, dependente da complementaridade entre a dupla fita. A plataforma mais usada de microarranjos de cDNA consiste na impressão de milhares de pontos, geralmente numa placa de vidro, que consistem em diferentes moléculas de DNA (como oligonucleotídeos, clones de cDNA ou produtos de PCR - as sondas) todas previamente conhecidas. Das amostras de interesse a serem comparadas (controle *versus* doente, por exemplo), o RNA é extraído, usado para a síntese de cDNA simples-fita e estes são marcados com diferentes fluoróforos (Cy-3 e Cy-5 geralmente). O cDNA marcado é hibridizado às sondas da placa de vidro. A intensidade dos sinais de hibridização é determinada em processo automatizado, com auxílio de computadores, de acordo com a intensidade da fluorescência obtida em cada ponto.

Microarranjos de cDNA comerciais podem revelar uma cópia de RNA num universo de 250.000 moléculas, conferindo a esta técnica a possibilidade de quantificar alguns do mRNAs de mais baixa abundância[130]. Em neuropsiquiatria, o uso do microarranjos de cDNA gerou muitos dados em diferentes grupos ao redor do mundo, muitos dos quais em SCZ, os quais serão mais extensamente discutidos nos capítulos seguintes.

[126] Kozal MJ, Shah N, Shen N, Yang R, Fucini R, Merigan TC, Richman DD, Morris D, Hubbell E, Chee M, Gingeras TR.Extensive polymorphisms observed in HIV-1 clade B protease gene using high-density oligonucleotide arrays.Nat Med. 1996 Jul;2(7):753-9.
[127] Günthard HF, Wong JK, Ignacio CC, Havlir DV, Richman DD.Comparative performance of high-density oligonucleotide sequencing and dideoxynucleotide sequencing of HIV type 1 pol from clinical samples.AIDS Res Hum Retroviruses. 1998 Jul 1;14(10):869-76
[128] Blencowe BJ. Alternative splicing: new insights from global analyses.Cell. 2006 Jul 14;126(1):37-47.)
[129] Hughes TR, Hiley SL, Saltzman AL, Babak T, Blencowe BJ.Microarray analysis of RNA processing and modification.Methods Enzymol. 2006;410:300-16
[130] Mirnics K, Middleton FA, Lewis DA, Levitt P. Analysis of complex brain disorders with gene expression microarrays: schizophrenia as a disease of the synapse. Trends Neurosci. 2001;8:479-486. Review.

1.2.3 Análise Seriada da Expressão Gênica (Serial Analysis of Gene Expression - SAGE)

Em 1995, Velculescu *et al*. desenvolveram uma poderosa ferramenta de quantificação da expressão gênica, denominada *Serial Analysis of Gene Expression* ou SAGE)[131], que permite a definição detalhada do perfil de expressão gênica em larga escala, onde cada transcrito é transformado numa pequena etiqueta ("tag"). Diferentes etiquetas são obtidas a partir dos diversos transcritos. Estas são concatenadas e clonadas em vetores de sequenciamento. Cada reação de sequenciamento permite a leitura de dezenas de *tags* e a contagem final destas permite inferir o número de cópias de cada transcrito na amostra original. Deste modo, se um número suficiente de *tags* é gerado, a freqüência de cada *tag* no conjunto total, gera um número absoluto que representa o nível de expressão do respectivo transcrito, evitando erros inter-experimentais inerentes à normalização. A forma padronizada característica dos experimentos de SAGE, que revela de forma absoluta quanto cada transcrito é observado em uma população total de transcritos, possibilita o acúmulo destes dados e a comparação entre diferentes bibliotecas, ou diferentes estudos[132,133].

A técnica de SAGE se apóia em 3 princípios básicos: primeiro, uma seqüência nucleotídica 10 pares de bases, a chamada *tag*, é gerada por uma enzima de restrição com um sítio freqüente. Supondo toda a variação possível em 10 nt, temos que 1.048.576 *tags* diferentes (ou 4^{10}) são possíveis, o que em teoria, seria suficiente para discriminar a maioria dos transcritos derivados do genoma humano[134]. O segundo princípio é que as *tags* podem ser concatenadas e sequenciadas em conjunto, sendo posteriormente separadas por análises de bioinformática. E terceiro, o sequenciamento de todas as *tags* previamente concatenadas possibilita que estas sejam contadas num universo total de *tags*, possibilitando assim uma quantificação absoluta de cada *tag* (ou cada transcrito) no conjunto total dos genes.

[131] Velculescu V. E., Zhang L., Vogelstein B., Kinzler K. W. Serial Analysis of Gene Expression; Science, 1995, 270; 484-487
[132] Madden SL, Wang CJ, Landes G. Serial analysis of gene expression: from gene discovery to target identification. Drug Discov Today 2000, 5: 415-25.
[133] Velculescu VE, Vogelstein B, Kinzler KW. Analysing uncharted transcriptomes with SAGE. Trends Genet 2000, 16: 423-5.
[134] Patino WD, Mian OY, Hwang PM. Serial analysis of gene expression: technical considerations and applications to cardiovascular biology. Circ Res. 2002 Oct 4;91(7):565-9.

A produção de uma biblioteca de SAGE envolve passos enzimáticos seqüenciais. O RNA da célula ou tecido de interesse deve ser isolado e imobilizado em esferas magnéticas associadas a oligo(dT), possibilitando a captura seletiva do RNA poli-A+ diretamente da amostra. O cDNA é então sintetizado a partir deste oligo(dT) e clivado com uma enzima de restrição com um sítio de corte freqüente (4 bases – a mais usada é a *NlaIII* que reconhece CATG). Após a digestão, são ligados adaptadores aos cDNAs, os quais contém um sítio para a enzima *BsmFI* além de um sítio complementar para um oligonucleotídeo de interesse. A enzima *BsmFI* reconhece o sítio de digestão (GGGAC) e cliva o cDNA 10 nt após este sítio, liberando uma pequena *tag*. Essas *tags* são pareadas formando '*ditags*', amplificadas por PCR, digeridas com *NlaIII*, ligadas para formar concatâmeros e clonadas em um vetor para o seqüenciamento (Figura 2).

Num experimento de SAGE típico, o sequenciamento de 2000 insertos pode produzir cerca de 50.000 *tags*, o que tem sido considerado como um número de detecção sensível para a maior parte dos genes diferencialmente regulados[135]. Além disso, em geral duas bibliotecas de SAGE são comparadas (controle *versus* caso) o que permite o cálculo da diferença de expressão gênica para cada transcrito com boa significância estatística, reduzindo a necessidade de replicatas. Muitos trabalhos utilizando SAGE já foram publicados visando a identificação de marcadores tumorais[136,137,138,139], a identificação de novos genes[140] e no estudo de outras doenças[141,142,143] ou para a análise de outros

[135] Ruijter JM, Van Kampen AH, Baas F. Statistical evaluation of SAGE libraries: consequences for experimental design. Physiol Genomics. 2002 Oct 29;11(2):37-44.
[136] Pauws E, Veenboer GJ, Smit JW, de Vijlder JJ, Morreau H, Ris-Stalpers C. Genes differentially expressed in thyroid carcinoma identified by comparison of SAGE expression profiles. FASEB J. 2004 Mar;18(3):560-1.
[137] Peters DG, Kudla DM, Deloia JA, Chu TJ, Fairfull L, Edwards RP, Ferrell RE. Comparative gene expression analysis of ovarian carcinoma and normal ovarian epithelium by serial analysis of gene expression. Cancer Epidemiol Biomarkers Prev. 2005 Jul;14(7):1717-23.
[138] Yang YQ, Zhang LJ, Dong H, Jiang CL, Zhu ZG, Wu JX, Wu YL, Han JS, Xiao HS, Gao HJ, Zhang QH. Upregulated expression of S100A6 in human gastric cancer. J Dig Dis. 2007 Nov;8(4):186-93.
[139] Abba MC, Sun H, Hawkins KA, Drake JA, Hu Y, Nunez MI, Gaddis S, Shi T, Horvath S, Sahin A, Aldaz CM. Breast cancer molecular signatures as determined by SAGE: correlation with lymph node status. Mol Cancer Res. 2007 Sep;5(9):881-90.
[140] Chen J, Sun M, Lee S, Zhou G, Rowley JD, Wang SM. Identifying novel transcripts and novel genes in the human genome by using novel SAGE tags. Proc Natl Acad Sci U S A 2002, 99: 12257-62.
[141] Patino WD, Mian OY, Kang JG, Matoba S, Bartlett LD, Holbrook B, Trout HH 3rd, Kozloff L, Hwang PM. Circulating transcriptome reveals markers of atherosclerosis. Proc Natl Acad Sci U S A. 2005 Mar 1;102(9):3423-8.
[142] Yang YQ, Zhang LJ, Dong H, Jiang CL, Zhu ZG, Wu JX, Wu YL, Han JS, Xiao HS, Gao HJ, Zhang QH. Upregulated expression of S100A6 in human gastric cancer. J Dig Dis. 2007 Nov;8(4):186-93.
[143] Shadeo A, Chari R, Lonergan KM, Pusic A, Miller D, Ehlen T, Van Niekerk D, Matisic J, Richards-Kortum R, Follen M, Guillaud M, Lam WL, Macaulay C. Up regulation in gene expression of chromatin remodelling factors in cervical intraepithelial neoplasia. BMC Genomics. 2008 Feb 4;9(1):64.

organismos[144,145,146]. Porém, alguns poucos trabalhos foram publicados em relação a doenças neuropsiquiátricas[147,148,149] sendo que até hoje não foi publicado nenhum artigo demonstrando o uso de SAGE no estudo da SCZ.

A natureza compreensiva e quantitativa dos dados de SAGE é interessante e aplicável na identificação de marcadores de doenças, incluindo SCZ. As principais vantagens e desvantagens de ESTs, microarranjos de cDNA e SAGE são destacadas na Tabela 5.

[144] Skuce PJ, Yaga R, Lainson FA, Knox DP.An evaluation of serial analysis of gene expression (SAGE) in the parasitic nematode, Haemonchus contortus. Parasitology. 2005 May;130(Pt 5):553-9.
[145] Ojopi EP, Oliveira PS, Nunes DN, Paquola A, DeMarco R, Gregório SP, Aires KA, Menck CF, Leite LC, Verjovski-Almeida S, Dias-Neto E.A quantitative view of the transcriptome of Schistosoma mansoni adult-worms using SAGE. BMC Genomics. 2007 Jun 21;8:186.
[146] Mihm M, Baker PJ, Fleming LM, Monteiro AM, O'Shaughnessy PJ. Differentiation of the bovine dominant follicle from the cohort upregulates mRNA expression for new tissue development genes. Reproduction. 2008 Feb;135(2):253-65.
[147] Sun Y, Zhang L, Johnston NL, Torrey EF, Yolken RH. Serial analysis of gene expression in the frontal cortex of patients with bipolar disorder. Br J Psychiatry Suppl. 2001 Jun;41:s137-41.
[148] Feldker DE, Datson NA, Veenema AH, Meulmeester E, de Kloet ER, Vreugdenhil E. Serial analysis of gene expression predicts structural differences in hippocampus of long attack latency and short attack latency mice. Eur J Neurosci. 2003 Jan;17(2):379-87.
[149] Ozbas-Gerçeker F, Redeker S, Boer K, Ozgüç M, Saygi S, Dalkara T, Soylemezoglu F, Akalan N, Baayen JC, Gorter JA, Aronica E. Serial analysis of gene expression in the hippocampus of patients with mesial temporal lobe epilepsy. Neuroscience. 2006;138(2):457-74.

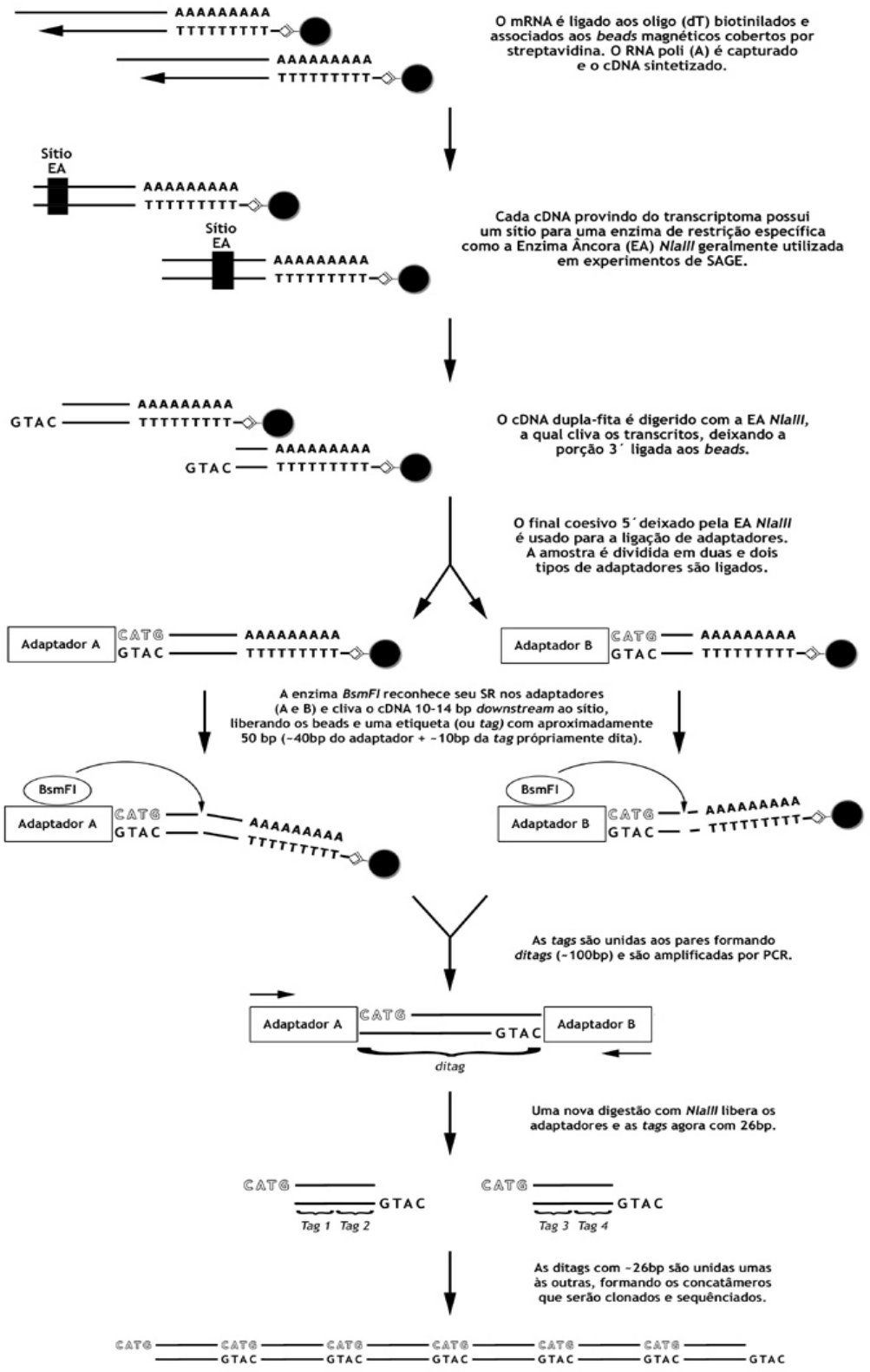

Figura 2: Esquema resumido dos procedimentos realizados durante a construção de uma biblioteca de SAGE.

Tabela 5: Vantagens e desvantagens mais relevantes das principais técnicas de estudo de expressão gênica em larga escala.

Técnica	*Vantagens Principais*	*Desvantagens Principais*
Análise de ESTs	- Maior conteúdo informativo (permite análises qualitativas e quantitativas). - Maior precisão na identificação de genes. - Excelente para gerar dados de alteração qualitativa de expressão gênica (como *splicing* alternativo). - Permite a análise de polimorfismos e mutações. - Excelente ferramenta para descoberta de novos genes.	- Preparação da biblioteca de cDNA pode ser complexa. - Grandes quantidades de RNA são requeridas nos protocolos padrão. - Alto custo para sequenciamento de um número significativo de ESTs. - Grande trabalho de bioinformática é requerido. - Método *low-throughput* com as técnicas padrão de sequenciamento.
Microarranjos de cDNA	- Custo relativamente baixo após a disponibilização dos equipamentos. - Permite análise em larga-escala de milhares de transcritos simultaneamente. - Método altamente automatizado. - É o método mais bem caracterizado para estudos de expressão gênica.	- Possui problemas de quantificação. - Permite somente a análise dos transcritos presentes nos microarranjos. - Não permite a análise de genes desconhecidos. - cDNA pode hibridizar uma sonda similar (problema ajustável) - Requer equipamentos especiais para análise dos microarranjos. - Dados de expressão são sempre relativos e inerentes à sonda e à plataforma usada. - Requer extensas análises de bioinformática.
SAGE	- Permite a análise de genes ainda não descritos. - Estima diretamente o nível de abundância absoluta da expressão de cada gene. - Não requer uma lista de genes candidatos. - Excelente capacidade de quantificação. - Não requer réplicas ou padronização. - Não requer equipamentos especiais. - Resultados de quaisquer experimentos de SAGE são diretamente comparáveis.	- *Tags* podem ser ambíguas. - Alguns transcritos podem não ter o sítio para a enzima âncora, impedindo sua avaliação. - Baixa detecção de transcritos raros. - Dificil e custoso trabalho de sequenciamento de DNA (quando se aplicam métodos tradicionais). - Requer recursos de bioinformática.

1.3 Análise da Expressão de Genes Específicos

Em geral, após uma avaliação em larga escala que aponta possíveis marcadores, os genes de interesse são quantificados de modo individual. Em 1977 foi desenvolvida uma técnica para detecção e quantificação de RNA que foi denominada como *Northern blot*[150] (um trocadilho em relação à técnica de blotting de DNA genômico, descrita pelo pesquisador Ed Southern, e denominada comumente de Southern blot). O princípio da técnica se baseia na hibridização de sondas específicas, marcadas por substâncias detectáveis (radioativamente, quimicamente ou imunologicamente, por exemplo), às moléculas de mRNA. Deste modo, no *Northern blot,* as moléculas de RNA são fracionadas por tamanho, geralmente em gel de agarose desnaturante, e transferidas para um suporte sólido (em geral, membranas de nylon ou nitrocelulose) onde são detectadas após a hibridização das sondas. *Northern blot* é ainda uma técnica muito utilizada, apesar do menor poder de quantificação e de exigir grandes quantidades de amostra.

Além do Northern blot a quantificação da expressão gênica pode ser feita por PCR semiquantitativa. Para isto, as moléculas RNA são transformadas em cDNA dupla-fita através da utilização da transcriptase reversa e posteriormente amplificadas pela técnica de Reação em Cadeia da Polimerase (RT-PCR). A intensidade das bandas é verificada em géis de agarose, por densitometria. Em geral, utiliza-se diferentes quantidades de ciclos na PCR, e o número de ciclos necessários para a visualização dos produtos, é inversamente proporcional ao número de cópias do mRNA. Porém há necessidade de grande otimização da técnica para que os dados possam ser mais confiáveis[151], e a reação nunca oferece resultados absolutos, apenas relativos.

[150] Alwine JC, Kemp DJ, Stark GR. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. Proc Natl Acad Sci U S A. 1977 Dec;74(12):5350-4.
[151] Ding C, Cantor CR. Quantitative analysis of nucleic acids--the last few years of progress. J Biochem Mol Biol. 2004 Jan 31;37(1):1-10.

1.3.1 Reação em Cadeia da Polimerase em Tempo Real (Real Time PCR)

Atualmente, a técnica mais sensível e confiável para a quantificação da expressão de um gene é a técnica de *Real-Time PCR* ou ainda PCR quantitativa (qPCR) que pode ser feita de duas maneiras: utilizando-se o fluoróforo *SYBR Green* ou a tecnologia *TaqMan*.

Com a utilização de qualquer um dos métodos, inicialmente o mRNA do gene de interesse é transformado em cDNA e este cDNA será submetido a uma reação de amplificação usando PCR, na presença de um fluoróforo do tipo *SYBR Green* ou uma sonda fluorescente no caso do *TaqMan*.

SYBR Green são pequenas moléculas capazes de se ligar especificamente ao DNA dupla-fita sem inibir a amplificação. Quando *SYBR Green* é adicionado à amostra, ele imediatamente liga-se a todos os DNA dupla-fita presentes no meio emitindo fluorescência. Durante a PCR, novas moléculas de DNA dupla-fita são geradas e moléculas de *SYBR Green* se ligam a estas. Com a amplificação exponencial do DNA, a fluorescência emitida pelo *SYBR Green* também aumenta exponencialmente.

Com a tecnologia de *TaqMan,* uma sonda específica para o gene de interesse se liga às moléculas alvo. Durante a amplificação, esta sonda é degradada pela atividade exonucleásica da DNA polimerase usada na PCR, liberando uma fluorescência captada pelo aparelho. Para isto, o termociclador usado na PCR em tempo real está acoplado a um sistema computadorizado capaz de captar e medir a fluorescência emitida durante a reação, em tempo real. Deste modo, à medida que os ciclos da PCR avançam a fluorescência aumenta exponencialmente, gerando uma curva sigmoidal característica, que corresponde à amplificação do gene-alvo. Esta curva pode ser comparada a outras curvas de fluorescência geradas pela amplificação do mesmo gene em outra amostra permitindo uma comparação precisa de sua expressão[152]. Para tornar o processo ainda mais preciso, são usados controles endógenos, que permitem a acurada normalização da fluorescência pela quantidade inicial de cDNA utilizado.

[152] Nolan T, Hands RE, Bustin SA. Quantification of mRNA using real-time RT-PCR.Nat. Protoc. 2006, 1: 1559-1582.

As vantagens da qPCR são a rapidez na geração dos resultados (cerca de 60 ou 120 minutos), a eliminação da necessidade de manipulação dos produtos de amplificação (o que reduz em muito os problemas de contaminação de PCR), o uso da sensível detecção dos *amplicons* por fluorescência que exige menor quantidade de amostra inicial e principalmente a especificidade (de modo especial quando são empregados sondas e iniciadores específicos), a sensibilidade (uma única cópia de um gene pode ser detectada de modo consistente) e a natureza quantitativa, que permite grande precisão, além do uso de controles endógenos múltiplos, que padronizam as quantidades iniciais de material[153]. É difícil encontrar hoje na Biologia Molecular técnica tão precisa e controlada como a qPCR. No entanto, entre as desvantagens estão a necessidade do uso de uma plataforma específica e o custo relativamente alto das reações, especialmente quando são usadas sondas gene-específicas, marcadas com fluoróforos[154].

1.4 Análises Proteômicas

Em 1995, Wilkins *et al.*[155,156,157,158], introduziram o termo "proteoma" como "o complemento protéico total do genoma". Ao passo que o genoma é relativamente estável, a natureza do proteoma é dinâmica alterando-se frente a diferentes condições e estímulos. Logo, o estudo global destas alterações representa uma forma de investigar e integrar processos em sistemas vivos para melhor entender o funcionamento de uma célula ou tecido ao nível molecular. Rapidamente, a essência do proteoma foi sendo desvendada levando até uma definição mais completa: proteoma é o conjunto de proteínas expressas por uma

[153] Wittwer CT, Herrmann MG, Gundry CN, Elenitoba-Johnson KS. Real-time multiplex PCR assays. Methods. 2001 Dec;25(4):430-42.
[154] Mackay IM. Real-time PCR in the microbiology laboratory. Clin Microbiol Infect. 2004 Mar;10(3):190-212.
[155] Wasinger VC, Cordwell SJ, Cerpa-Poljak A, Yan JX, Gooley AA, Wilkins MR, Duncan MW, Harris R, Williams KL, Humphery-Smith I.Progress with gene-product mapping of the Mollicutes: Mycoplasma genitalium.Electrophoresis. 1995 Jul;16(7):1090-4.
[156] Wilkins MR, Sanchez JC, Gooley AA, Appel RD, Humphery-Smith I, Hochstrasser DF, Williams KL. Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. Biotechnol Genet Eng Rev. 1996;13:19-50.
[157] Wilkins MR, Pasquali C, Appel RD, Ou K, Golaz O, Sanchez JC, Yan JX, Gooley AA, Hughes G, Humphery-Smith I, Williams KL, Hochstrasser DF. From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and amino acid analysis. Biotechnology (N Y). 1996 Jan;14(1):61-5.
[158] Williams KL, Hochstrasser DF Introduction to the proteome. Em: Proteome Research: New Frontiers in Functional Genomics (Wilkins MR, Williams KL, Apple RD, Hochstrasser DF. eds.), Springer, 1997, pp. 1-12.

célula, tecido ou organismo em determinado momento, sob determinada condição.

O reconhecimento da importância estratégica do proteoma para uma maior compreensão bioquímica dos organismos e dos processos biológicos, aliado ao desenvolvimento de abordagens de estudo do conjunto de proteínas de uma amostra permitiu o surgimento da "proteômica", que visa estudar também a estrutura[159,160], função[161,162] e o controle dos sistemas biológicos pela análise das várias propriedades das proteínas. A proteômica abrange a sequencia (identidade), abundância, atividade e estrutura das proteínas expressas por uma célula, bem como modificações, interações e translocações sofridas por cada proteína.

Durante a segunda metade dos anos 90, a identificação e quantificação sistemática de todas as proteínas detectáveis em um organismo ou tecido era o objetivo inicial central numa análise proteômica. Atualmente, as análises comparativas são mais empregadas, visto o interesse de revelar proteínas diferencialmente expressas, diante de seu potencial como marcador terapêutico e que sirva para diagnóstico.

Apesar do avanço rápido e contínuo das tecnologias para o estudo cada vez mais ágil e preciso do proteoma, uma das técnicas ainda mais empregadas para estes estudos é a combinação da eletroforese de duas dimensões em gel de poliacrilamida (2-DE) para separação, detecção e quantificação das proteínas, juntamente com a posterior identificação destas por espectrometria de massas (MS). A combinação da 2-DE com a MS foi por anos a base dos estudos de proteoma[163] e continua sendo uma das ferramentas mais utilizadas. Apesar da técnica de 2-DE ter sido inicialmente introduzida na década de 70[164,165], a

[159] Norin M, Sundström M. Structural proteomics: developments in structure-to-function predictions. Trends Biotechnol. 2002 Feb;20(2):79-84.
[160] Sali A, Glaeser R, Earnest T, Baumeister W. From words to literature in structural proteomics.Nature. 2003 Mar 13;422(6928):216-25.
[161] Uetz P, Giot L, Cagney G, Mansfield TA, Judson RS, Knight JR, Lockshon D, Narayan V, Srinivasan M, Pochart P, Qureshi-Emili A, Li Y, Godwin B, Conover D, Kalbfleisch T, Vijayadamodar G, Yang M, Johnston M, Fields S, Rothberg JM. A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae. Nature. 2000 Feb 10;403(6770):623-7..
[162] Ito T, Chiba T, Ozawa R, Yoshida M, Hattori M, Sakaki Y. A comprehensive two-hybrid analysis to explore the yeast protein interactome. Proc Natl Acad Sci U S A. 2001 Apr 10;98(8):4569-74.
[163] Klose J, Nock C, Herrmann M, Stühler K, Marcus K, Blüggel M, Krause E, Schalkwyk LC, Rastan S, Brown SD, Büssow K, Himmelbauer H, Lehrach H. Genetic analysis of the mouse brain proteome. Nat Genet. 2002 Apr;30(4):385-93.
[164] Klose, J. Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals. Humangenetik 1975, 26, 231-243.

maioria dos métodos que possibilitou identificar proteínas separadas em 2-DE de forma rápida, conclusiva e com sensibilidade foi desenvolvida apenas nos últimos 15 anos[166]. Os principais fatores que possibilitaram o surgimento destes métodos foram avanços na técnica de espectrometria de massas, a disponibilidade de sequencias genômicas completas e o desenvolvimento de programas computacionais para correlação dos dados obtidos por espectrometria de massa com os bancos de dados de sequencias.

1.4.1 Eletroforese de Duas Dimensões em Gel de Poliacrilamida

A eletroforese de duas dimensões em gel de poliacrilamida (2-DE) é um dos mais poderosos métodos disponíveis para separar misturas complexas de proteínas[167,168] e apesar do surgimento de técnicas recentes como "shotgun proteomics" e também das conhecidas limitações que apresenta, tais como a exclusão de proteínas muito pequenas (< 6000 Da), muito grandes (> 120000 Da), muito ácidas (pI < 3.5), muito básicas (pI > 9) e muito hidrofóbicas[169] e a não detecção de proteínas com baixos níveis de expressão[170], esta técnica ainda é uma das ferramentas mais utilizadas nos estudos de proteoma para detecção e identificação de proteínas com potencial de biomarcadores.

A 2-DE é constituída essencialmente de dois passos: O primeiro, a chamada primeira dimensão, é a focalização isoelétrica ou isoeletrofocalização (IEF), na qual as proteínas são separadas em um gradiente de pH até alcançarem à posição estacionária onde a carga total é zero (ou seja, seu ponto isoelétrico - pI). O segundo passo, chamada segunda dimensão, as proteínas previamente separadas pela IEF são novamente separadas por eletroforese do tipo SDS-PAGE. Essa separação é baseada no peso molecular (MW) das proteínas. O resultado é um perfil onde cada proteína representa um ponto, comumente chamado de "spot", sendo que quanto maior o volume do *spot* maior a concentração da proteína.

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[165] O'Farrel, P. H. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 1975, 250, 4007-4021.
[166] Aebersold, R., Goodlett, D. Mass spectrometry in Proteomics. Chem. Rev. 2001, 101, 269-295.
[167] Westermeier R. (1997) Electrophoresis in Practice. VCH, Weinheim, Alemanha.
[168] Wilkins MR; Williams KL; Appel RD e Hochstrasser (1997) eds: Proteome Research: New Frontiers in Functional Genomics.Springer-Verlag Berlin Heidelberg, Alemanha. Pag 1-11.
[169] Gygi SP, Corthals GL, Zhang Y, Rochon Y, Aebersold R. Evaluation of two-dimensional gel electrophoresis based proteome analysis tecnology. Proc. Natl. Acad. Sci. 2000 15:97(17): 9390-9395
[170] Futcher B, Latter GI, Monardo P, McLaughlin CS, Garrels JI. A Sampling of the Yeast Proteome. MCB 1999; Vol 19 (11): 7357-7376.

Segundo um sistema cartesiano, da esquerda para a direita há um aumento do pI e de baixo para cima um aumento da MW. A alta resolução da 2-DE resulta do fato da primeira e segunda dimensões serem baseadas em parâmetros independentes (pI e massa molecular das proteínas) (Figura 3).

As imagens dos perfis 2-DE obtidas experimentalmente são digitalizadas para subseqüentes análises quantitativas e qualitativas, realização de calibrações de pI e MW, quantificações dos *spots* e comparações automáticas de diferentes géis utilizando-se programas computacionais especializados para detecção de proteínas diferencialmente expressas.

A 2-DE é um poderoso método de separação por ser uma plataforma que isola e de certa forma purifica a maioria das proteínas expressas em uma amostra, simultânea e bi-dimensionalmente. Ainda, provém informações sobre o pI, MW e abundância relativa de cada proteína, estando estas ainda viáveis para análises pós-separação, como por exemplo espectrometria de massas. Através da 2-DE é possível ainda visualizar algumas modificações pós-traducionais através da mobilidade eletroforética alterada de alguns conjuntos de proteínas.

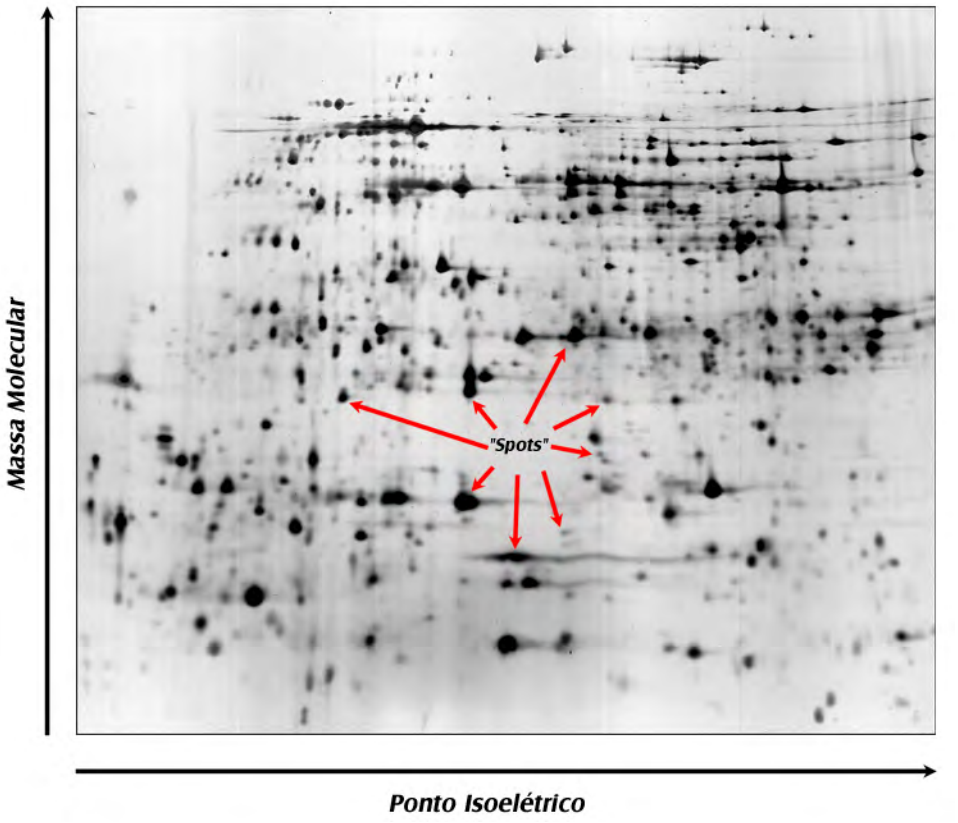

Figura 3: Gel 2-DE. Plano cartesiano onde os pares ordenados representam os spots*, que são, teoricamente, proteínas purificadas da célula ou tecido em questão. As setas indicam um aumento gradativo do pI e do MW.*

1.4.1.1 Detecção e Quantificação das Proteínas Separadas por 2-DE

As proteínas separadas por 2-DE podem ser detectadas por diversos métodos colorimétricos, fluorescentes ou radiográficos pré ou pós-eletroforese[171,172]. O método de detecção escolhido é um importante determinante da sensibilidade e da qualidade da quantificação.

Os métodos mais comuns utilizados são colorimétricos reversíveis pós-eletroforese, dentre eles a coloração com coomassie brillant blue (CBB) ou nitrato de prata (Ag). Ambos têm inúmeros protocolos descritos e são simples, de baixo custo e compatíveis com a espectrometria de massas para análises subseqüentes.

[171] Patton WF. Detection technologies in proteome analysis. J Chromatogr B Analyt Technol Biomed Life Sci. 2002 May 5;771(1-2):3-31.
[172] Miller I, Crawford J, Gianazza E. Protein stains for proteomic applications: which, when, why? Proteomics. 2006 Oct;6(20):5385-408.

A coloração por Ag[173,174] é o método colorimétrico pós-eletroforese mais sensível existente (1-10 ng/*spot*), porém de difícil quantificação visto que a intensidade da coloração é saturável, depende do tempo de revelação, e não oferece grande linearidade entre a intensidade do *spot* e a quantidade de uma determinada proteína, tornando difícil uma comparação mais precisa, especialmente entre diferentes géis[175,176]. O príncipio é saturar o gel com íons de prata que vão interagir com as proteínas por uma ligação metálica e reduzir a prata ligada às proteínas a sua forma metálica. Previamente à redução da prata, o gel é lavado para retirar o excesso dos íons e desfazer as ligações prata-matriz.

A coloração com CBB[177] é o método colorimétrico pós-eletroforese mais confiável para quantificação dos *spots* já que apresenta linearidade quantitativa na faixa de concentração de proteína de 10-100 ng/*spot*[178]. O corante coomassie (R ou G) liga-se às proteínas por interação eletrostática ou hidrofóbica em meio ácido. Entretanto, a sensibilidade é 5 a 50 vezes mais baixa que coloração por Ag se usado um protocolo de CBB coloidal[179] (utilizando como corante o Coomassie-G250) ou um protocolo de CBB simples (utilizando como corante o Coomassie-R250) respectivamente.

Os métodos radioativos e fluorimétricos são bastante sensíveis e quantitativamente muito precisos. Os fluorimétricos ganharam maior confiança dos pesquisadores principalmente depois do lançamento comercial das técnicas de detecção pré-eletroforese denominadas SYPRO[180] e gel de eletroforese diferencial (difference gel electrophoresis - DIGE)[181,182] cuja sensibilidade chega

[173] Switzer RC 3rd, Merril CR, Shifrin S.A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels.Anal Biochem. 1979 Sep 15;98(1):231-7.
[174] Merril CR, Switzer RC, Van Keuren ML.Trace polypeptides in cellular extracts and human body fluids detected by two-dimensional electrophoresis and a highly sensitive silver stain.Proc Natl Acad Sci U S A. 1979 Sep;76(9):4335-9.
[175] Rabilloud, T. Mechanisms of protein silver staining in polyacrylamide gels: a 10-year synthesis. Electrophoresis 1990, 11, 785-794.
[176] Rabilloud, T. Silver staining of 2-D electrophoresis gels. Methods Mol. Biol. 1999, 112, 297-305.
[177] Fazekas de St. Groth S, Webster RG, and Datyner A. Two new staining procedures for quantitative estimation of proteins on electrophoretic strips. Biochim. Biophys. Acta 1963, 71: 377–391.
[178] Poland J, Rabilloud T, Sinha P. in: Walker, J.M. (Ed.), The Proteomics Handbook, Humana Press, Totowa 2005.
[179] Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, Orecchia P, Zardi L, Righetti PG. Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis. 2004 May;25(9):1327-33.
[180] Steinberg, T. H., Jones, L. J., Haugland, R. P., Singer, V. L. SYPRO orange and SYPRO red protein gel stains: one-step fluorescent staining of denaturing gels for detection of nanogram levels of protein. Anal. Biochem. 1996, 239, 223-237.
[181] Unlü M, Morgan ME, Minden JS. Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis. 1997 Oct;18(11):2071-7.
[182] Tonge R, Shaw J, Middleton B, Rowlinson R, Rayner S, Young J, Pognan F, Hawkins E, Currie I, Davison M.Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology.Proteomics. 2001 Mar;1(3):377-96.

a 0,2 ng/*spot*[183]. Porém, a necessidade de equipamentos especiais para a detecção dos *spots* e o uso de fluoróforos sensíveis e de alto custo são desvantagens do método. Além disso, normalmente há a necessidade de se realizar um experimento colorimétrico para a identificação das proteínas detectadas por estes métodos, já que as proteínas previamente marcadas com os fluoróforos não são passíveis de identificação por MS.

Qualquer que seja a forma de detecção, a imagem do gel é digitalizada para que seja feita a análise quantitativa das proteínas detectadas através de programas computacionais específicos para análises de perfis 2-DE. Os programas mais utilizados são Image Master Elite e Image Master Platinum (GE Biosciences), Melanie (desenvolvido pela Geneva Bioinformatics, hoje produto da GE Biosciences) e PDQuest (BioRad), que contém algoritmos capazes de detectar os *spots*, quantificar os *spots* através de seu volume relativo, eliminar possíveis artefatos que, apesar de apresentar cor, não sejam propriamente proteínas e calibrar o pI e MW dos géis de acordo com a faixa de pI e porosidade do gel utilizada. Determinar o pI e MW é importante visto que estes parâmetros são essenciais para a confirmação da identidade da proteína posteriormente identificada por MS.

Nestes softwares ainda é possível comparar diferentes amostras de um mesmo tipo celular em diferentes condições, através da sobreposição dos perfis 2-DE: os *spots* correspondentes nos diferentes géis são detectados e seus volumes comparados a fim de se estabelecer uma relação da expressão de cada uma das proteínas detectadas. Este processo de comparação dos volumes dos *spots* é conhecido como análise densitométrica.

É importante destacar que o sucesso da análise de imagem depende fundamentalmente da qualidade e reprodutibilidade dos perfis 2-DE a serem comparados, principalmente nos géis onde métodos colorimétricos são aplicados. Esta variável é descartada quando métodos fluorimétricos são utilizados, visto que diferentes amostras são simultaneamente separadas num mesmo gel.

[183] Sitek B, Scheibe B, Jung K, Schramm A, Stuehler K Difference Gel Electrophoresis (DIGE): the Next Generation of Two-Dimensional Gel Electrophoresis for Clinical Research. In: Marcus K, Stuehler K, van Hall A, Hamacher M et al. (Eds.), Proteomics in Drug Research, Wiley-VCH, Weinheim 2006, pp. 33–55.

1.4.2 Identificação de Proteínas por Espectrometria de Massas do tipo MALDI-TOF E MALDI-TOF/TOF

Mesmo sendo a 2-DE uma poderosa ferramenta analítica, esta técnica somente permite detectar proteínas, atribuindo a elas pI e MW, mas não é capaz de identificá-las. Ao visualizar um perfil 2-DE, não temos como saber *a priori* quais as proteínas representadas em cada um dos *spots*. Porém, quando a MS é integrada como método pós-2-DE, uma ferramenta poderosa se configura já que as proteínas correspondentes aos *spots* selecionados podem ser identificadas. Contudo, para esta ferramenta funcionar de maneira completa, é necessário conhecer o genoma completo do organismo em questão.

A fusão da 2-DE, MS com os dados de um genoma, permite a identificação das proteínas de um *spot* pelo método "impressão digital das massas de peptídeos" (*peptide-mass finger printing* - PMF) [184,185,186,187,188]. Cada proteína tem uma seqüência específica de aminoácidos. Quando uma protease de ação conhecida atua sobre determinada proteína, peptídeos são gerados, cada um com uma seqüência e uma massa específica. Esta mesma protease quando atua em outra proteína, gera também peptídeos, mas com massas e seqüências distintas da primeira. Diante das possíveis combinações de aminoácidos, peptídeos distintos são gerados após a ação da protease usada, gerando uma "impressão digital" para cada proteína.

O fato de cada proteína ter sua "impressão digital" é a base da técnica de PMF: Na prática, os *spots* (que em geral representam uma única proteína) dos perfis 2-DE são recortados do gel, tripsinizados e os peptídeos resultantes da digestão tem suas massas medidas por MS. O resultado final é uma lista de massas de peptídeos que deve ser, teoricamente, única para cada proteína. Paralelamente, todas as seqüências das proteínas conhecidas para o organismo

[184] Mann, M., Hojrup, P., Roepstorff, P. Use of mass spectrometric molecular weight information to identify proteins in sequence databases. Biol. Mass Spectrom. 1993, 22, 338-345.
[185] Henzel WJ, Billeci TM, Stults JT, Wong SC, Grimley C, Watanabe C. Identifying proteins from two-dimensional gels by molecular mass searching of peptide fragments in protein sequence databases. Proc Natl Acad Sci U S A. 1993 Jun 1;90(11):5011-5.
[186] James, P., Quadroni, M., Carafoli, E., Gonnet G. Protein identification by mass profile fingerprinting. Biochem. Biophys. Res. Commun. 1993, 195, 58-64.
[187] Yates JR 3rd, Speicher S, Griffin PR, Hunkapiller T. Peptide mass maps: a highly informative approach to protein identification. Anal Biochem. 1993 Nov 1;214(2):397-408.
[188] Thiede B, Höhenwarter W, Krah A, Mattow J, Schmid M, Schmidt F, Jungblut PR. Peptide mass fingerprinting. Methods. 2005 Mar;35(3):237-47.

em questão (provindas de um banco de dados genômico) são clivadas *in silico* gerando uma "impressão digital virtual". Os dados obtidos experimentalmente são cruzados com os dados *in silico* possibilitando a identificação do gene produtor daquela proteína através de programas específicos, e a consequente identificação dos *spots* de interesse (Figura 4).

Existem atualmente diversos tipos de espectrômetros de massas. Normalmente, para aplicação da técnica de PMF, usam-se aparelhos do tipo MALDI-TOF (*matrix-assisted laser desorption/ionization-time of flight* - desorção e ionização por laser assistida por matriz - tempo de vôo).

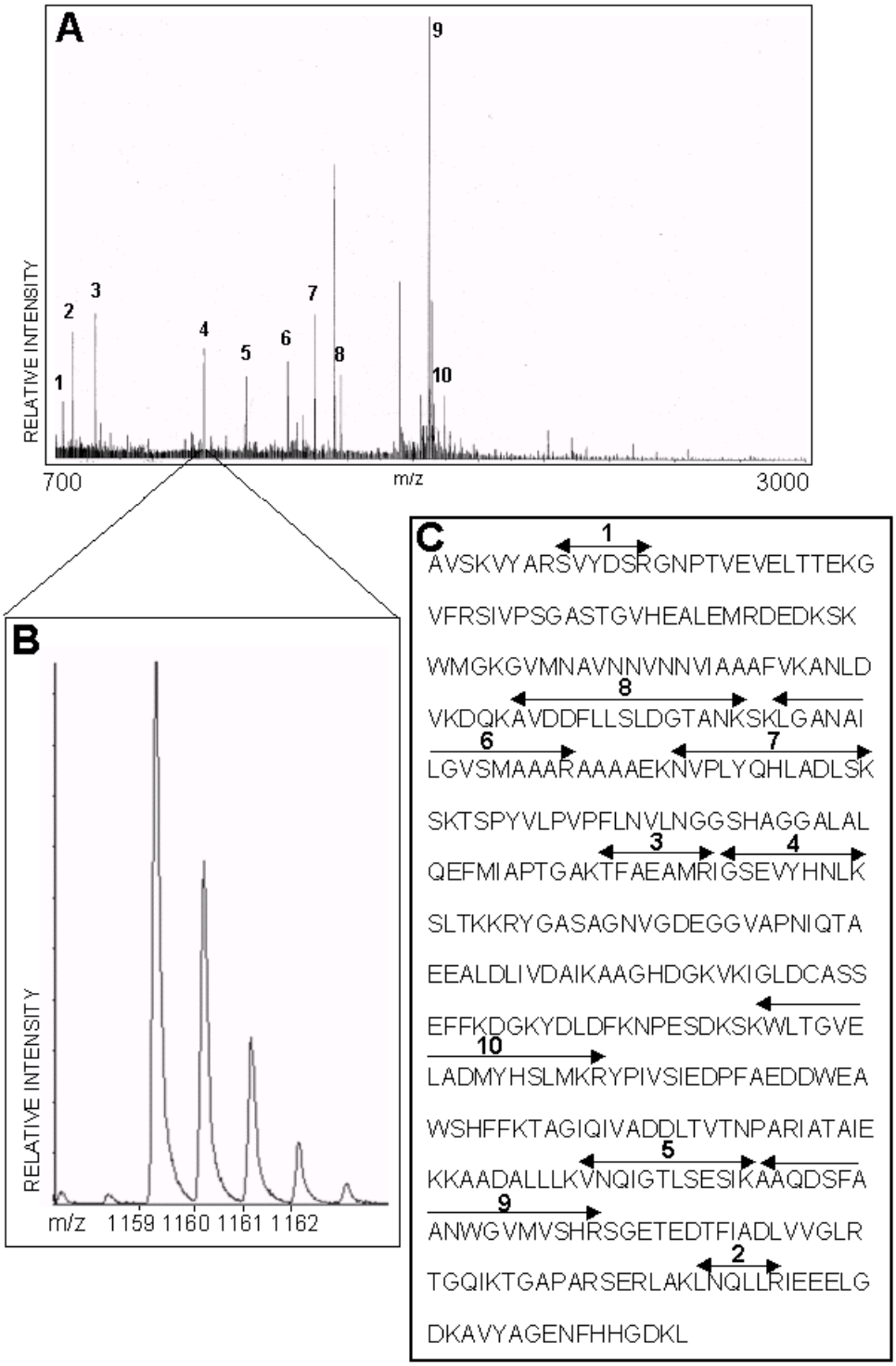

Figura 4: Mapeamento de peptídios da enzima enolase. O spot *separado por 2-DE foi digerido com tripsina e os peptídios resultantes analisados em espectrômetro de massa MALDI-TOF. Procura em bancos de dados indicou a proteína como sendo enolase 2. (A) Espectro de massa dos peptídios trípticos. Os números indicam os peptídios correspondentes na Figura 4C. (B) Ampliação da região entre 1158 e 1164Da mostrando o conjunto de isótopos do peptídio com massa de 1159.66Da. (C) Seqüência de amino ácidos da enolase 2. Note que a tripsina cliva na parte C-terminal da lisina (K) e arginina (R).*

Um espectrômetro de massas (EM) pode ser considerado uma "balança molecular", um equipamento cuja finalidade é medir a massa de átomos e moléculas com extrema precisão.

Um EM é basicamente dividido em três partes: um ionizador, um analisador e um detector, sendo que a molécula a ser analisada percorre obrigatoriamente este caminho nesta ordem. O ionizador é a parte do EM responsável por ionizar as moléculas pois somente nesta condição elas serão analisadas. No analisador sua massa é medida e o detector capta a informação do analisador.

Num EM MALDI-TOF, MALDI é o método de ionização utilizado. Sucintamente, as moléculas a serem analisadas são misturadas a uma matriz, um composto que tem grande capacidade de absorver energia. O EM tem um canhão de laser (fonte de energia) que atinge o complexo matriz-molécula. A absorção da energia por este complexo transforma-o em um gás ionizado. Logo, a molécula em questão está pronta para ser analisada. TOF é o método de análise deste EM. A sigla significa "tempo de vôo" já que as moléculas agora ionizadas vão literalmente "voar" por um tubo analítico que tem comprimento conhecido, um portão de entrada e um portão de saída. Estabelece-se então uma relação da massa com o tempo que a molécula leva para atravessar o tubo. Uma molécula de 500 Da passa mais rapidamente pelo tubo se comparada a uma molécula de 1000 Da, que voa mais devagar pelo tubo. A relação do tamanho do tubo com o tempo exato que a molécula leva para atravessá-lo determina sua massa com grande precisão. O detector é a parte do EM que vai colher os dados analisados e passar isso para o programa computacional do aparelho. Inúmeras moléculas podem ser analisadas no mesmo experimento, ou seja, peptídeos provenientes da digestão tríptica de um *spot* são todos analisados simultaneamente (Figura 5). Qualquer que seja o EM utilizado, todos geram espectros de massa que são representados como um gráfico de duas dimensões contendo valores de m/z no eixo x e intensidades relativas no eixo y (Figura 4A). Nos espectros gerados em instrumentos de alta resolução, cada peptídeo aparece como um conjunto de isótopos (Figura 4B), conseqüência do fato de que 1.1 % dos carbonos naturalmente existente são o isotopicamente pesado 13C. Como os peptídeos

contêm grandes quantidades de átomos de carbono, uma fração significativa dos peptídeos irão conter um 13C (e um valor de massa aumentado em 1 unidade de massa comparado ao íon contendo apenas 12C), ou dois 13C (e um valor de massa aumentado em 2 unidades de massa comparado ao íon contendo apenas 12C), e assim por diante.

MALDI é um método desenvolvido pelos alemães Franz Hillenkamp and Michael Karas[189,190] a partir dos conceitos de ionização branda de moléculas por laser introduzidos em 1985 (somente publicados em 1988)[191] Sr. Koichi Tanaka, laureado com o Prêmio Nobel de Química em 2002.

Dependendo do organismo em questão, a complexidade do genoma pode gerar proteínas que contenham "impressões digitais" semelhantes, dificultando sua identificação precisa. Isso eventualmente acontece nas análises do proteoma de mamíferos para algumas proteínas. Nestes casos, mais dados da proteína são necessários para sua a identificação. Inicialmente, para a resolução destes problemas, os EM MALDI-TOF passaram a executar análises do tipo "post-source decay" (PSD)[192,193] que permite selecionar no espectro de massas peptídeos para que estes sejam fragmentados numa câmara de colisão com gás. Os fragmentos, dos mais diferentes tamanhos, desde o peptídeo até um simples aminoácido têm as massas medidas e então a seqüência de aminoácidos do peptídeo é resolvida, gerando um dado muito mais robusto à identificação da proteína. Equipamentos mais sofisticados foram desenvolvidos e os EM híbridos (são os EM conhecidos como MS/MS), que contém mais de um analisador em série, tornaram máquinas poderosas na resolução de dados das moléculas analisadas. Um equipamento muito utilizado em estudos de proteoma e que se encaixa bem de acordo com as necessidades de estudos como o que desenvolvemos é o MALDI-TOF/TOF, onde dois analisadores TOF estão presentes no mesmo EM sendo o primeiro para a

[189] Karas, M.; Bachmann, D.; Hillenkamp, F. "Influence of the Wavelength in High-Irradiance Ultraviolet Laser Desorption Mass Spectrometry of Organic Molecules". Anal. Chem. 1985, 57: 2935-9.
[190] Karas M, Hillenkamp F. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons". Anal. Chem. 1988, 60 (20): 2299-301.
[191] Tanaka K, Waki H, Ido Y, Akita S, Yoshida Y, Yoshida T. Protein and Polymer Analyses up to m/z 100 000 by Laser Ionization Time-of flight Mass Spectrometry Rapid Comm Mass Spectrom 1988, 2 (20): 151-3.
[192] Kaufmann R, Spengler B, Lützenkirchen F. Mass spectrometric sequencing of linear peptides by product-ion analysis in a reflectron time-of-flight mass spectrometer using matrix-assisted laser desorption ionization. Rapid Commun Mass Spectrom. 1993 Oct;7(10):902-10.
[193] Gevaert K, Vandekerckhove J. Protein identification methods in proteomics. Electrophoresis. 2000 Apr;21(6):1145-54.

obtenção do PMF e o segundo para medir a massa dos fragmentos dos peptídeos selecionados, gerando a seqüência de aminoácidos destes[194,195].

Uma descrição mais detalhada dos tipos de EM mais usados, suas capacidades e limitações e análises MS/MS, bem como os algoritmos usados para procura em bancos de dados visando a identificação das proteínas pode ser encontrada nas revisões[196,197,198].

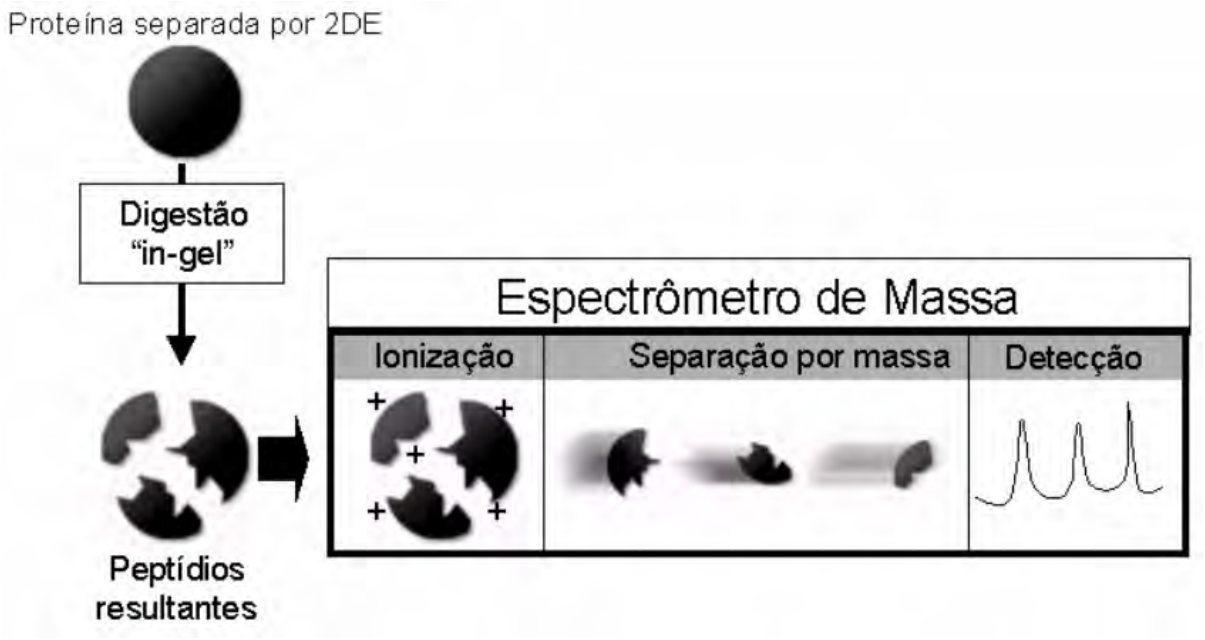

Figura 5: Espectrometria de massas do tipo MALDI-TOF. A proteína separada por 2-DE é digerida in gel (geralmente com tripsina), as massas dos peptídios resultantes são determinadas em espectrômetro MALDI-TOF. Nos EM híbridos MALDI-TOF/TOF, os picos de massa referentes aos peptídeos a serem analisados são selecionados para análise da seqüência de aminoácidos daqueles peptídeos.

[194] Medzihradszky KF, Campbell JM, Baldwin MA, Falick AM, Juhasz P, Vestal ML, Burlingame AL. The characteristics of peptide collision-induced dissociation using a high-performance MALDI-TOF/TOF tandem mass spectrometer. Anal Chem. 2000 Feb 1;72(3):552-8.
[195] Bienvenut WV, Déon C, Pasquarello C, Campbell JM, Sanchez JC, Vestal ML, Hochstrasser DF. Matrix-assisted laser desorption/ionization-tandem mass spectrometry with high resolution and sensitivity for identification and characterization of proteins. Proteomics. 2002 Jul;2(7):868-76.
[196] Patterson, S. D., Aebersold, R. Mass spectrometric approaches for the identification of gel-separated proteins. Electrophoresis 1995, 16, 1791-1814.
[197] Aebersold R, Mann M.Mass spectrometry-based proteomics. Nature. 2003 Mar 13;422(6928):198-207.
[198] Domon B, Aebersold R. Mass spectrometry and protein analysis. Science. 2006 Apr 14;312(5771):212-7.

1.4.3 "Shotgun Proteomics"

Primeiramente descrita em 1999[199], a idéia básica de *shotgun* seria a digestão de um proteoma usando enzimas específicas, seguida da separação dos peptídeos resultantes por sistemas de cromatrografia multidimensional seguida de análise por MS/MS (de qualquer natureza), a fim de eliminar ao máximo as limitações eletroforéticas, desde a solubilidade das proteínas na 2-DE[200] até a representação fiel de todas elas (muito ou pouco expressas)[169]. Algoritmos computacionais específicos permitiriam então a interpretação dos espectros de massas e identificação das proteínas, visto a complexidade dos dados gerados[201,202]. Atualmente, é uma técnica cada vez mais empregada nos estudos de proteoma e tende em longo prazo a substituir a 2-DE, apesar de alguns defenderem que o uso integrado de ambas seria a melhor opção[203,204].

Qualitativamente uma rotina *shotgun* como a acima descrita seria ideal para estudos de proteoma. Porém, quantitativamente, no caso de um proteoma comparativo por exemplo, esta abordagem não seria tão completa já que os espectros de massa dos peptídeos em si não são bons quantificadores da expressão protéica. Para uma quantificação adequada num proteoma comparativo por *shotgun*, há a necessidade de inserção na rotina *shotgun* de métodos quantitativos precisos.

Sempre houve entre os pesquisadores de proteoma uma preocupação com a exatidão na quantificação diferencial da expressão protéica[205]. A quantificação da expressão diferencial em perfis 2-DE é feita de acordo com o volume dos *spots*. Porém, um novo conceito de quantificação da expressão protéica revolucionou a quantificação proteômica com a introdução do ICAT (Isotope

[199] Link AJ, Eng J, Schieltz DM, Carmack E, Mize GJ, Morris DR, Garvik BM, Yates JR 3rd. Direct analysis of protein complexes using mass spectrometry. Nat Biotechnol. 1999 Jul;17(7):676-82.
[200] Adessi C, Miege C, Albrieux C, Rabilloud T. Two-dimensional electrophoresis of membrane proteins: a current challenge for immobilized pH gradients. Electrophoresis. 1997 Jan;18(1):127-35.
[201] Wu CC, MacCoss MJ. Shotgun proteomics: tools for the analysis of complex biological systems. Curr Opin Mol Ther. 2002 Jun;4(3):242-50.
[202] MacCoss MJ. Computational analysis of shotgun proteomics data. Curr Opin Chem Biol. 2005 Feb;9(1):88-94.
[203] Li S, Wang J, Zhang X, Ren Y, Wang N, Zhao K, Chen X, Zhao C, Li X, Shao J, Yin J, West MB, Xu N, Liu S. Proteomic characterization of two snake venoms: Naja naja atra and Agkistrodon halys. Biochem J. 2004 Nov 15;384(Pt 1):119-27.
[204] Kubota K, Kosaka T, Ichikawa K. Combination of two-dimensional electrophoresis and shotgun peptide sequencing in comparative proteomics. J Chromatogr B Analyt Technol Biomed Life Sci. 2005 Feb 5;815(1-2):3-9.
[205] Gygi SP, Rist B, Aebersold R. Measuring gene expression by quantitative proteome analysis. Curr Opin Biotechnol. 2000 Aug;11(4):396-401.

Coded Afinity Tags)[206]. O reagente ICAT, conhecido como isótopo estável, consiste de três componentes funcionais: O primeiro componente se liga em cisteínas reduzidas, o segundo componente pode conter átomos de deutério (ICAT pesado) ou não (ICAT leve - 8 Da mais "leve" que o ICAT pesado) e o terceiro componente serve para o isolamento seletivo dos peptídios marcados com ICAT. A idéia é ligar a uma determinada amostra o "ICAT leve" e à amostra a ser comparada o "ICAT pesado", misturar as amostras, digerir as proteínas e medir a massa dos peptídeos resultantes. O resultado é um espetro de massas onde duplas de picos com 8 Da de diferença representam o mesmo peptídeo das duas diferentes amostras, sendo que a razão das intensidades absolutas dos picos de massas determinam a expressão protéica.

Como toda técnica, ICAT tem suas limitações (não permite a análise de proteínas sem cisteína, e ainda, como a análise é feita em peptídios gerados pela digestão de proteínas, importantes informações geralmente analisáveis em proteínas intactas (como presença de isoformas e modificações pós-traducionais) são perdidas) e isso fez com que novos isótopos estáveis com mesmo conceito surgissem, dentre eles Global Internal Standard Technology (GIST)[207] and Isobaric Tags for Relative and Absolute Quantification (iTRAQ)[208].

A introdução destes reagentes à rotinas *shotgun* resolve de maneira muito elegante e precisa o problema da quantificação supracitado. O uso destes isótopos pode ainda ser combinado com a 2-DE.

O conceito de *shotgun proteomics* não define um sistema único de trabalho: é possível combinar diferentes técnicas, dentre elas cromatografias de diversos tipos e o uso de isótopo estáveis, para se estabelecer um sistema de trabalho robusto e confiável. Um tipo de rotina *shotgun* pode ser visto na Figura 6.

[206] Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat Biotechnol. 1999 Oct;17(10):994-9.
[207] Goodlett DR, Keller A, Watts JD, Newitt R, Yi EC, Purvine S, Eng JK, von Haller P, Aebersold R, Kolker E. Differential stable isotope labeling of peptides for quantitation and de novo sequence derivation. Rapid Commun Mass Spectrom. 2001;15(14):1214-21.
[208] Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlet-Jones M, He F, Jacobson A, Pappin DJ. Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol Cell Proteomics. 2004 Dec;3(12):1154-69.

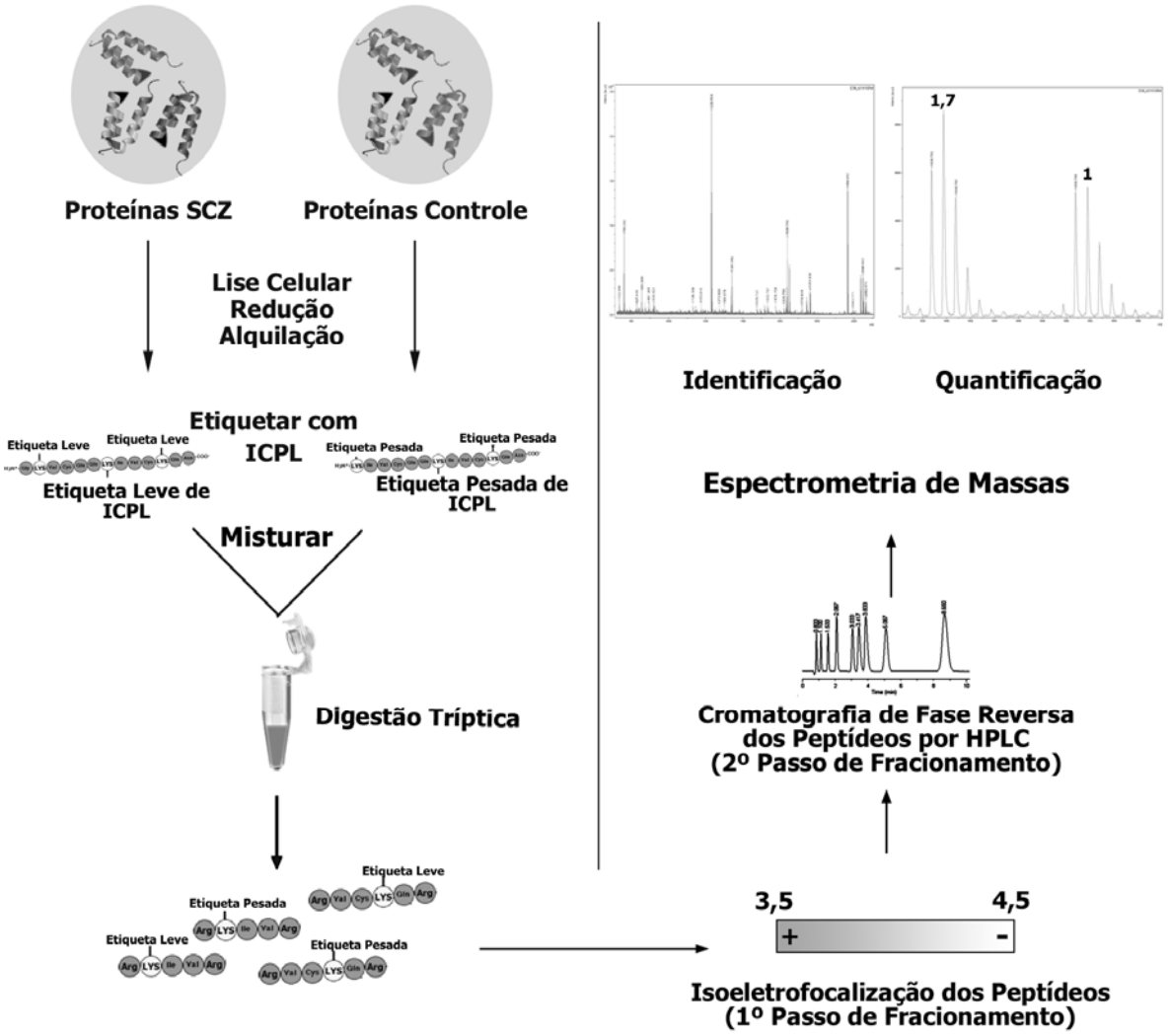

Figura 6: Esquema de funcionamento de uma técnica de shotgun *com a introdução do método ICPL para quantificação das proteínas, sendo SCZ e Controle as amostras exemplo.*

1.4.4 ICPL (Isotope-Coded Protein Labeling)

ICPL é um novo isótopo apresentado em 2005[209] cujo conceito é o mesmo do ICAT, porém com algumas vantagens. Os isótopos ICPL são etiquetas de ácido succínico nicotínico (Figura 7), onde o isótopo "leve" é composto de carbonos normais (carbonos 12) e a etiqueta "pesada" é constituída do isótopo 13 de carbono. Estas etiquetas ligam-se às proteínas em resíduos -NH2 reativos (resíduos de lisina ou N-terminais de proteínas). Basicamente, as proteínas de interesse são rotuladas separadamente, uma com um isótopo leve e outra com um isótopo pesado. As amostras são então misturadas e aplicadas a uma matriz de separação (2-DE ou *Shotgun*). Num perfil 2-DE por exemplo, um *spot* será formado por proteínas provindas de ambas as amostras, algumas rotuladas com o isótopo leve, outras com o isótopo pesado. Diferença que somente será perceptível quando estas proteínas passarem para uma plataforma MS, já que os picos de massa apareceram duplos, com 8 Da de diferença. A quantificação das proteínas se dá pela relação direta do valor da intensidade dos picos provindos do isótopo leve e do pesado, representando fielmente a quantidade de proteínas rotulada com cada isótopo.

ICPL tem as vantagens de: permitir o fracionamento da amostra ao nível protéico com ambas amostras inseridas em somente um experimento, o que evita diferenças experimentais, aumentando a eficácia do comparativo de proteoma; a digestão das proteínas é feita simultâneamente com as duas amostras em um único tubo, eliminando diferenças experimentais; ICPL permite o fracionamento em nível de peptídeos, o que pode aumentar a sensibilidade dos espectros de massas, podendo assim identificar um maior número de proteínas da amostra; devido a características químicas, a etiqueta promove melhor ionização dos peptídeos, aumentando a sensibilidade dos espectros; a quantificação é eficazmente feita pela intensidade dos picos de massas obtidas por espectrometria de massas tipo MALDI e a identificação das proteínas pode ser feita por MALDI MS-MS.

[209] Schmidt A, Kellermann J, Lottspeich F. A novel strategy for quantitative proteomics using isotope-coded protein labels. Proteomics. 2005 Jan;5(1):4-15. Erratum in: Proteomics. 2005 Feb;5(3):826.

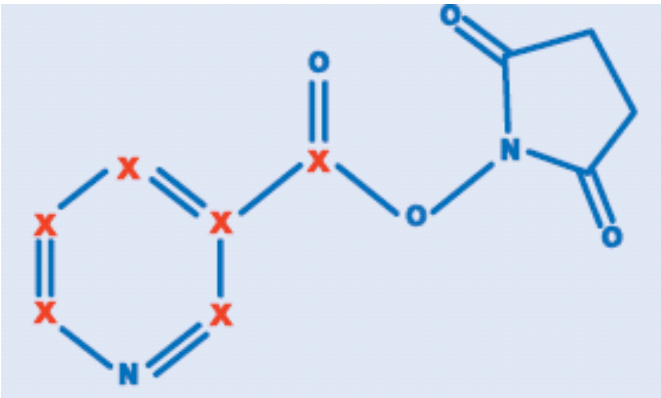

Figura 7: A etiqueta ICPL. Os "X" representam os carbonos que são 12 ou 13 de acordo com o isótopo.

1.5 Estudo de transcriptoma e proteoma em esquizofrenia

Estudos de transcriptoma revelam importantes informações tanto sobre a regulação da expressão gênica como sobre a compreensão sistemática celular através de tecnologias muito precisas. Porem, tão importante quanto às informações do transcriptoma são as informações do proteoma visto que, mesmo que uma proteína não possa ser sintetizada sem seu mRNA correspondente, é possível que uma proteína esteja na célula quando seu mRNA não está mais presente e, ainda, é possível haver uma expressão aumentada ou diminuída de mRNA em determinada situação que não resulte em alteração na mesma escala da proteína codificada. Ou ainda, determinado mRNA pode estar em mesmo nível de expressão enquanto que a expressão protéica pode estar aumentada ou diminuída. Todas estas situações são devidas aos mecanismos regulatórios pós-transcricionais e pós-traducionais que regulação a expressão gênica e protéica que fazem com que a quantidade de proteína não seja necessariamente proporcional à quantidade de seu mRNA correspondente[210]. Esta afirmação é confirmada por estudos que compararam dados de transcriptoma e proteoma e encontraram pequena sobreposição de genes e proteínas diferencialmente expressas. Ao analisar os efeitos do tratamento com rapamicina sobre uma linhagem de células-T, Grolleau *et al*. revelaram 136 genes e 111 proteínas diferencialmente expressas. No entanto, a sobreposição transcriptoma-proteoma

[210] Unwin RD, Whetton AD. Systematic proteome and transcriptome analysis of stem cell populations. Cell Cycle. 2006 Aug;5(15):1587-91.

foi de apenas 15 genes[211]. De modo similar, Bro *et al.*, estudando leveduras tratadas com lítio, identificaram 48 proteínas diferencialmente expressas das quais 17 genes corresponderam em alteração no transcriptoma[212]. Estes dados confirmam que transcriptoma e proteoma compartilham informações estritamente complementares, clamando então a importância do estudo de ambas, principalmente se juntas.

Sendo a SCZ ainda uma doença não totalmente elucidada em diversos aspectos, apesar de todos os esforços provindos de diversas áreas do conhecimento, a determinação do transcriptoma e do proteoma diferencialmente reguladas em indivíduos com SCZ utilizando-se das técnicas acima descritas, pode fornecer uma abordagem convergente para a identificação de genes e proteínas de suscetibilidade específicos, além de potenciais alvos terapêuticos.

[211] Grolleau A, Bowman J, Pradet-Balade B, Puravs E, Hanash S, Garcia-Sanz JA, Beretta L. Global and specific translational control by rapamycin in T cells uncovered by microarrays and proteomics. J Biol Chem 2002; 277:22175-84.
[212] Bro C, Regenberg B, Lagniel G, Labarre J, Montero-Lomeli M, Nielsen J. Transcriptional, proteomic, and metabolic responses to lithium in galactose-grown yeast cells. J Biol Chem 2003; 278:32141-9.

Objetivos

* Comparar o perfil transcricional de córtex pré-frontal de pacientes com SCZ com o perfil transcricional de controles livres de distúrbios neuropsiquiátricos utilizando SAGE.

* Confirmar os genes diferencialmente expressos utilizando qPCR.

* Comparar o proteoma do córtex pré-frontal de pacientes com SCZ com o proteoma de controles livres de distúrbios neuropsiquiátricos utilizando a combinação 2-DE/MS e *shotgun proteomics*.

* Comparar o proteoma do lobo temporal anterior de pacientes com SCZ com o proteoma de controles livres de distúrbios neuropsiquiátricos utilizando *shotgun proteomics*.

* Cruzar os dados de transcriptoma e proteoma buscando genes e proteínas que possam estar envolvidos com a SCZ apontando para mecanismos envolvidos na patogênese da doença além de possíveis alvos moleculares em potencial para o desenvolvimento de drogas mais eficazes e específicas.

Justificativa

Estudos têm demonstrado que não existe um gene ou proteína em especial que pode ser o principal causador da SCZ e sim que existem alterações em alguns genes, em nível de polimorfismos de DNA ou com expressão gênica anormal ou ainda que haja um conjunto de proteínas sendo diferencialmente expressas que acabam por modular outros genes e proteínas, formando assim um conjunto de eventos integrados que, somados a componentes ambientais, geram a doença. Uma abordagem simultânea envolvendo expressão gênica e análise proteômica nunca foi feita em SCZ.

SAGE permite determinar o perfil de expressão dos transcritos de um tecido de forma precisa e detalhada, porém, sabe-se que a expressão gênica em RNA não é linear à produção de proteínas. Justifica-se então a grande importância da análise do proteoma utilizando plataformas de última geração.

Conclui-se então que, analisar de forma global a expressão gênica e de proteínas de cérebros de pacientes com SCZ em comparação a cérebros de pacientes livres de SCZ, permitiria a descoberta de alterações em conjuntos de genes e de proteínas diferencialmente expressos na doença e em condições normais. O intercruzamento destes dados fornecerá bases moleculares para o entendimento da doença.

Organização Desta Tese

Os resultados e discussões obtidos por esta tese estão apresentados em capítulos em acordo com os manuscritos publicados e redigidos.

No primeiro capitulo, descrevemos os dados de expressão gênica global do córtex pré-frontal de cérebros de pacientes com esquizofrenia, obtidos por SAGE bem como a confirmação da expressão de alguns genes em amostras individuais, obtidos por qPCR.

O segundo traz o capítulo "RNA Biomarkers in Schizophrenia" do livro "Biomarkers for Psychiatric Disorders" a ser publicado na Alemanha pela editora 'Springer', com edição do Prof. Dr. Christoph W. Turck.

O terceiro capítulo descreve através de um artigo publicado os métodos de padronização por nós estabelecidos para a ideal determinação do proteoma de amostras de cérebros humanos.

No quarto capítulo, através de um manuscrito a ser submetido, revelamos os dados de proteoma do córtex pré-frontal de cérebros de pacientes com esquizofrenia, obtidos por Eletroforese de Duas Dimensões em Gel de Poliacrilamida e Espectrometria de Massas.

No quinto capítulo, através de um manuscrito submetido, revelamos os dados de proteoma do lobo temporal anterior de cérebros de pacientes com esquizofrenia, obtidos pela metodologia de *Shotgun Proteomics*.

O sexto capítulo mostra sob a forma de um manuscrito submetido o proteoma de córtex pré-frontal de indivíduos livres de doenças neuropsiquiátricas.

E finalmente, no sétimo capítulo, apresentamos os dados prévios do proteoma de córtex pré-frontal de cérebros de pacientes com esquizofrenia pela metodologia de *Shotgun Proteomics.*

Capítulo 1:

Análise da Expressão Gênica de Cérebros de Pacientes com Esquizofrenia e Controles Utilizando SAGE e Real-Time PCR.

1) Introdução:

1.1) Esquizofrenia e estudos genéticos

Como apresentado na introdução desta tese, a esquizofrenia (SCZ) é provavelmente causada por uma série de variações polimórficas em genes de "pequeno efeito" que, em conjunto com importantes e variados fatores ambientais, resulta em alterações de neurogênese e má formação do sistema nervoso central (SNC) precursores de alterações cognitivas e funcionais.

Muitas das abordagens genéticas na busca dos genes envolvidos na SCZ são basicamente dependentes da identificação de polimorfismos genéticos. Isto ocorre, principalmente, pela grande dificuldade em se obter material biológico adequado para avaliar a expressão gênica.

1.2) Amostras de tecido cerebral de pacientes SCZ

Graças a uma colaboração entre o Laboratório de Neurociências (LIM-27) do Instituto de Psiquiatria da Faculdade de Medicina da Universidade de São Paulo, dirigido pelo Prof. Dr. Wagner F. Gattaz e o Zentralinstitut für Seelische Gesendheit (Instituto Central de Saúde Mental) em Mannheim, Alemanha através da Dra. Andrea Schmitt, dispusemos de amostras cerebrais derivadas do córtex cerebral pré-frontal de indivíduos com SCZ, além amostras-controle pareadas, oriundas de indivíduos livres de histórico neuropsiquiátrico.

As amostras utilizadas neste trabalho foram derivadas de 14 pacientes crônicos portadores de SCZ e 10 indivíduos usados como controles. Tais amostras foram coletadas após assinatura, pelos familiares, de um termo de consentimento esclarecido previamente aprovado pelos comitês de ética em pesquisa envolvidos com a coleta do material na Alemanha. As amostras nos foram enviadas em gelo seco, para estudo no Brasil, após a macro-dissecção de regiões neuroanatômicas determinadas.

2) Objetivos e justificativa

Neste projeto, investigamos pela primeira vez o transcriptoma de cérebros de pacientes portadores de SCZ utilizando SAGE (*Serial Analysis of Gene*

Expression)[1]. Esta abordagem foi pioneira, pois ainda não existem trabalhos na literatura demonstrando o uso de SAGE na análise da expressão gênica em esquizofrenia. A escolha de SAGE foi feita devido a certas vantagens inerentes ao método, tal como a precisa quantificação absoluta dos transcritos, análise de genes não conhecidos, e o não requerimento de equipamentos especiais. Maiores detalhes da técnica de SAGE podem ser encontrados no ítem 1.2.3 da introdução desta tese.

A aplicação de uma técnica inédita para este tipo de amostras tem grande valor, pois a eventual identificação convergente de genes relacionados à SCZ, derivados através de diferentes abordagens técnicas, permite a construção de listas de marcadores mais consistentes, potencialmente úteis como alvos terapêuticos, além de contribuir na elucidação da patogênese da SCZ.

3) Resultados e discussão:

3.1) Amostras

3.1.1) Extração de RNA de amostras de tecido cerebral de pacientes esquizofrênicos e controles.

Devido à preciosidade das amostras e do alto custo envolvido com a preparação e sequenciamento das bibliotecas SAGE, a definição das amostras a serem utilizadas foi baseada principalmente nas análises feitas para determinar a integridade do material biológico. Deste modo, apenas amostras de RNA com suficiente nível de integridade foram utilizadas, segundo análises no *Agilent 2100 Bioanalyzer* (Agilent Technologies - Santa Clara - EUA). Um segundo parâmetro empregado para definir as amostras a serem utilizadas na confecção das bibliotecas SAGE foi a possibilidade de pareamento entre os sexos e as idades. Deste modo, buscamos que os dois grupos experimentais (SCZ e CTRL) fossem compostos pela mesma proporção entre homens e mulheres de mesma faixa etária. Deste modo, para a construção de duas bibliotecas SAGE, utilizamos 8 amostras (das 14 iniciais) provindas de pacientes e 8 amostras (das 10 iniciais) provindas dos controles.

[1] Velculescu V. E., Zhang L., Vogelstein B., Kinzler K. W. Serial Analysis of Gene Expression; Science, 1995, 270; 484-487.

Das amostras utilizadas de córtex pré-frontal dos cérebros provindos de pacientes com SCZ, a média de idade dos pacientes femininos era de 77,75 (variando entre 64 e 94 anos) e dos pacientes masculinos era de 67,5 (variando entre 43 e 81 anos). A coleta destas amostras foi feita em um intervalo post-mortem médio de 18,25 horas. Para as amostras controle, a média de idade dos pacientes femininos era de 79,66 (variando entre 66 e 91 anos) enquanto que a média etária para o sexo masculino foi de 66,66 (variando entre 53 e 79 anos), com um intervalo post-mortem médio de 22,16 horas.

Tabela 1: Dados clínicos dos pacientes e controles utilizados nas bibliotecas SAGE (em negrito) e nas análises por qPCR (todos os descritos). Abreviações: *atyptyp:* tempo de duração de tratamento com antipsicotico atípico (neurolepticos); *CPE:* medicação calculada em equivalentes de clorpromazina (mg); Anos de *CPE:* a somatória de medicamentos durante os últimos 10 anos em kg; *Hosp:* Tempo de hospitalização; *ECT:* Terapia eletroconvulsiva.

Amostra	Caso	Idade (Anos)	Gênero	*Post mortem* (horas)	Tipo de SCZ	Tempo da doença (Anos)	Tempo de medicação (Anos)	atyptyp	CPE	Anos de CPE	Causa da Morte	DSM IV	Idade de diagnóstico	Ultima Medicação	Uso de Cigarro	Uso de Alcool	Hosp (Anos)	ECT
13/00	SCZ	64	F	11	Residual, Episódios Paranóicos Crônicos	48	45	3	1536	7,7	Insuficiência Pulmonar	295.6	16	Clozapina 500 mg, Haloperidol 40 mg, Ciatil 40 mg	0	Não	21	Sim
36/02	SCZ	73	M	20	Residual, Episódios Paranóicos Crônicos	43	40	1	507,4	1,7	Infarto do Miocárdio	295.6	30	Perfenazina 32 mg, Prometazina 150 mg	30/ dia	Não	33	Não
39/02	SCZ	43	M	18	Residual, Episódios Paranóicos Crônicos	22	20	2	464	2,6	Infarto do Miocárdio	295.6	20	Zuclopetixol 40 mg, Valproato 1200 mg, Tiaprida 300 mg	0	Não	13	Não
39/03	SCZ	77	F	32	Residual, Episódios Paranóicos Crônicos	49	48	2	2555	8,3	Embolia Pulmonar	295.6	28	Clozapina 400 mg, Benperidol 25 mg, Clorprotixen 150 mg	0	Não	48	Sim
43/03	SCZ	76	F	17	Residual, Episódios Paranóicos Crônicos	49	47	1	300	4,9	Insuficiência Cardio-Pulmonar	295.6	27	Perazina 300 mg	0	Não	30	Sim
46/00	SCZ	63	F	31	Residual, Episódios Paranóicos Crônicos	40	30	3	75	1,8	Infarto do Miocárdio	295.6	24	Olanzapina 15 mg	30/ dia	Não	30	Sim
50/01	SCZ	81	M	4	Residual, Episódios Paranóicos Crônicos	62	50	1	92,8	1,4	Insuficiência Cardio-Pulmonar	295.6	19	Haloperidol 4 mg, Protipendil 80 mg	20/ dia	Não	48	Não
75/02	SCZ	92	F	37	Residual, Episódios Paranóicos Crônicos	51	48	1	100	3,4	Carcinoma Pancreático.	295.6	41	Protipendil 160 mg, Perazina 100 mg	0	Não	51	Não
83/01	SCZ	71	M	28	Residual, Episódios Paranóicos Crônicos	40	35	1	782,4	10	Infarto do Miocárdio	295.6	30	Haloperidol 32 mg, Pipamperona 40 mg	40/ dia	Não	12	Não
02/02	Controle	41	M	7							Infarto do Miocárdio				0	Não		
43/01	Controle	91	F	16							Insuficiência Cardio-Pulmonar				0	Não		
50/02	Controle	69	F	96							Embolia Pulmonar				0	Não		
51/02	Controle	57	M	24							Infarto do Miocárdio				0	Não		
57/02	Controle	53	M	18							Infarto do Miocárdio				0	Não		
59/02	Controle	63	M	13							Infarto do Miocárdio				0	Não		
61/01	Controle	66	M	16							Infarto do Miocárdio				0	Não		
72/02	Controle	79	M	24							Infarto do Miocárdio				0	Não		
27/02	Controle	55	M	19							Infarto do Miocárdio				20/ dia	Não		
81/01	Controle	76	F	17							Infarto do Miocárdio				10/ dia	Não		

3.1.2) Análise de 'pools'

Para cada amostra cerebral estudada (tanto dos pacientes quanto dos controles), isolamos, em média 60 µg de RNA. Para a preparação de cada uma das bibliotecas SAGE utilizamos um total de 152 µg de RNA (19µg de cada amostra). Optamos por analisar nossas amostras em *pools* por duas razões: primeiro, SAGE é uma abordagem extremamente poderosa, porém de grande complexidade técnica e de alto custo, o que tornaria inviável uma análise individual de cada amostra por tal técnica. Também, uma análise em *pool* para cada um dos grupos experimentais, pensando num possível diagnóstico molecular e por SCZ ser uma doença multifatorial, nos permite apontar alterações de expressão gênica inerentes à doença, diluindo a variabilidade inter individual sobre os resultados finais, apontando de forma mais consistente uma alteração de expressão em genes, famílias gênicas e vias metabólicas específicas. Além disto, a quantidade de material recebida foi um importante fator limitante. Estas amostras estavam vinculadas a vários projetos de pesquisa, e deveríamos utilizar a menor quantidade possível de material biológico em nossas análises.

3.2) Análise das bibliotecas de SAGE de tecido cerebral de pacientes esquizofrênicos e controles.

Na construção da biblioteca do *pool* de amostras de pacientes com SCZ (SCZ-lib) geramos um total de 51.661 *tags* (SCZ *tags*), enquanto que na construção da biblioteca do *pool* de amostras de controles pareados (CTRL-lib) geramos um total de 46.134 *tags* (CTRL *tags*). Ambas as bibliotecas possibilitaram a identificação de mais de 20.000 transcritos diferentes.

Uma análise geral preliminar mostra que as *tags* derivadas das duas bibliotecas tiveram um comportamento similar. Do total de SCZ *tags*, 5.373 (10,4%) foram observadas pelo menos 2 vezes e uma grande parte (85,92%) ocorreu em baixas freqüências (<10). Do total de CTRL *tags*, 4.834 (10,5%) foram observadas pelo menos 2 vezes e uma grande parte (87,86%) ocorreu em baixas freqüências (<10).

Uma análise destes dados, feita inicialmente com o software SAGE2000 (Invitrogen, Carlsbad, EUA) revelou 93 genes diferencialmente expressos (P<0.001), como apresentado na Tabela 2. Dos 93 genes diferencialmente expressos, 43 tinham uma expressão aumentada na SCZ-lib, enquanto que os 50 restantes tinham uma expressão reduzida. Os transcritos representados por *tags* sequenciadas uma única vez não foram considerados.

Os genes diferencialmente expressos foram classificados de acordo com os processos biológicos que participam utilizando a ferramenta Gene Ontology (www.geneontology.org).

Tabela 2: Genes identificados como diferencialmente expressos por análise de SAGE entre SCZ-lib e CTRL-lib normalizadas para 50.000 *tags*. Os genes são listados de acordo com os processos biológicos nos quais as respectivas proteínas estão envolvidas. O ponto de corte atribuído foi P<0,01. Estão indicados em itálico os genes que tiveram a expressão diferencial confirmada por real-time PCR (qPCR).

Biological Process	*Reg. in SCZ*	*Tags in SCZ*	*Tags in CTRL*	*Gene name*	*Protein Name (HPRD)*	*Chr Loci*	*Tag position*	*Tag_Sequence*	*AVG_P_Chance*
Signal transduction/Cell communication	↑	83	22	YWHAH	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide	22q12.3	1638	TCAATCAAGA	0
** Cell adhesion*	↓	58	125	CCNB1IP1	Cyclin B1 interacting protein 1	14q11.2	1370	CCACTGCACT	0
*** Regulation of cell cycle*	↓	73	139	MT3	Metallothionein 3 (growth inhibitory factor (neurotrophic))	16q13	383	AACAGCAAAA	0
	↓	282	373	NRGN	Neurogranin (protein kinase C substrate, RC3)	11q24	1058	TGACTGTGCT	0
	↓	13	40	*CSRP1*	Cysteine and glycine-rich protein 1	1q32	1757	GGCTGTACCC	0
	↓	47	84	PEBP1	Prostatic binding protein	12q24.23	1344	GGGGTAAGAA	4.67E-05
	↓	0	11	PPP3CA	Protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform (calcineurin A alpha)	4q21-q24	3178	AACTTAAAAA	2.73E-04
	↑	171	100	DNM1	Dynamin 1	9q34	3117	GGGGTGCTGT	4.77E-04
	↑	18	2	TERF2IP	Telomeric repeat binding factor 2, interacting protein	16q23.1	1315	GTAGGTGAGG	4.80E-04
	↑	20	3	GNAS	Guanine nucleotide binding protein G(S), alpha subunit	20q13.3	2291	ATTAACAAAG	5.33E-04
	↑	168	100	DPYSL2	Collapsin response mediator protein 2	8p22-p21	4438	GAACGCCTAA	7.23E-04
	↓	14	34	S100B	S100 calcium binding protein, beta (neural)	21q22.3	641	GCCGTGTAGA	8.37E-04
	↑	107	57	RPSA	* Laminin receptor 1 (ribosomal protein SA, 67kDa)	3p22.2	1000	GAAAAATGGT	9.17E-04
	↓	4	17	CALM1	Calmodulin 1 (phosphorylase kinase, delta)	14q24-q31	951	CAGCTTGACG	1.55E-03
	↓	6	20	GRIN2C	Glutamate receptor, ionotropic, N-methyl D-aspartate 2C	17q25		GTGACCACGG	2.22E-03
	↓	21	39	CLSTN1	Calsyntenin 1	1p36.22	5173	ATAGGTCAGA	4.24E-03
	↑	58	28	TSPAN7	Transmembrane 4 superfamily member 2	Xp11.4	1696	CCAACAAGAA	4.51E-03
	↓	0	7	RAB13	RAB13, member RAS oncogene family	1q21.2	1118	GTCTTTCTTG	5.28E-03
	↓	0	7	GRB2	** Growth factor receptor-bound protein 2	17q24-q25	3153	AAGCCTTGCT	5.28E-03
	↓	0	7	PPP2R5D	Protein phosphatase 2, regulatory subunit B (B56), delta isoform	6p21.1	2880/2860	CTAAGGTGGG	5.28E-03
	↓	0	7	TRIM23	ADP ribosylation factor domain protein 1	5q12.3		AGAAGGCCTT	5.28E-03
	↑	11	1	CAMK2B	Calcium/calmodulin-dependent protein kinase (CaM kinase) II beta	7p14.3-p14.1		ACCCCTAAAG	5.75E-03
	↑	30	11	MAP2K2	Mitogen-activated protein kinase kinase 2	19p13.3	1604	CAGGAACGGG	5.92E-03
	↑	19	5	MAP2K4	Mitogen-activated protein kinase kinase 4	17p11.2	3667	GAATCGAAGT	7.86E-03
Oligodendrocyte Metabolism	↓	1	6	MBP	Myelin Basic Protein	18q23	1322	GTGGCCCCAG	0
	↓	0	3	PMP22	Peripheral myelin protein 22	17p12-p11.2	386	ATCCTGTCGA	0
	↓	0	2	PMP2	Peripheral myelin protein 2	8q21.3-q22.1	3336	ATTGAAAAAA	0
	↓	0	2	MOBP	Myelin-associated oligodendrocyte basic protein	3p22.1	2952	ATTGCCAACA	0
	↓	0	2	LOC650177	Similar to sphingomyelinase	3p12.3	1525	GTGGGGAGGA	0
	↑	2	0	SMPD1	Sphingomyelin phosphodiesterase 1, acid lysosomal (acid sphingomyelinase)	11p15.4-p15.1	2313	GAGTAGAGGC	0
	↑	8	5	SMPD4	Sphingomyelin phosphodiesterase 4, neutral membrane (neutral	2q21.1	4169	TCCTTGCTTC	0

					sphingomyelinase-3)				
	↑	5	3	MAG	Myelin associated glycoprotein	19q13.1	2303	AAATAAATGC	0
Cell growth/maintenance	↑	139	68	TUBA1B	Tubulin, alpha, ubiquitous (K-ALPHA-1)	12q13.12	1564	TGTACCTGTA	0.00002
	↓	3	22	*KIF1C*	Kinesin family member 1C	17p13.2	4157	AGGATGTGGG	2.33E-05
	↑	27	7	SPTBN4	Spectrin, beta, non-erythrocytic 4	19q13.13	8658	GCCGCCTGGA	1.36E-03
	↑	10	0	SYNPO	Synaptopodin	5q33.1		TTCTCAATAC	1.57E-03
	↑	8	0	MIB2	Skeletrophin	1p36.33	3059	CGCCCCCTGC	5.68E-03
	↑	13	2	TUBB3	Tubulin, beta 3	16q24.3	1294	AACGACCTGG	6.83E-03
Metabolism/Energy pathways	↑	25	4	NDUFA1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1, 7.5kDa	Xq24	337	CAATGTGTTA	1.90E-04
	↑	102	50	MDH1	Malate dehydrogenase 1, NAD (soluble)	2p13.3	1227	CTGTTAGTGT	2.33E-04
	↑	44	15	ATP5A1	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1	18q12-q21	1825	AAAAATAAAG	4.63E-04
	↓	0	9	PDXK	Pyridoxal (pyridoxine, vitamin B6) kinase	21q22.3		GCGGTAAAAA	1.25E-03
	↑	120	72	GAPD	Glyceraldehyde-3-phosphate dehydrogenase	12p13	1232	TACCATCAAT	4.55E-03
	↑	8	0	DLD	Pyruvate dehydrogenase component E3	7q31-q32	1928	GCTGGAGCTA	5.68E-03
	↑	68	36	ALDOA	Aldolase A, fructose-bisphosphate	16q22-q24	947	GCGACCGTCA	6.01E-03
Regulation of nucleobase, nucleoside,	↓	1	19	STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)	17q21.31		GTGGCTCACG	0
nucleotide and nucleic acid metabolism	↓	0	14	ZNF664	Zinc finger protein ZFOC1	12q24.31	4971	CTGTTGCTGG	4.67E-05
	↓	13	32	ZNF446	Hypothetical protein FLJ20626	19q13.43	1770	GCCTGGGCTG	9.40E-04
	↑	8	0	SUB1	Activated RNA polymerase II transcription cofactor 4	5p13.3	1246	GGATGATGTC	5.68E-03
	↓	1	9	RBMY1F	RNA binding motif protein, Y-linked, family 1, member F	Yq11.223	1870/1873	TACTGCAAAA	6.82E-03
	↓	14	28	MBD3	Methyl-CpG binding domain protein 3	19p13.3	2330	GGTGGATGTG	8.67E-03
	↑	10	1	BLOC1S1	Biogenesis of lysosome-related organelles complex-1, subunit 1	12q13-q14	413	CGCACCATTG	9.90E-03
	↑	10	1	MYCBP2	MYC binding protein 2	13q22	14751	TCAATAAAAC	9.90E-03
Protein metabolism	↓	8	28	HSPCB	Heat shock 90kDa protein 1, beta	6p12	2306	GGCTCCCACT	1.67E-04
	↑	47	16	EIF4A2	Eukaryotic translation initiation factor 4A, isoform 2	3q28	1844	TCTTAATGAA	3.27E-04
	↑	156	95	RPL21	Ribosomal protein L21	13q12.2	502	GCATAATAGG	1.76E-03
	↑	50	21	RPS29	Ribosomal protein S29	14q	228	ATAATTCTTT	1.88E-03
	↓	77	105	RPLP2	Ribosomal protein, large P2	11p15.5-p15.4	403	GGATTTGGCC	2.71E-03
	↑	35	13	CPE	Carboxypeptidase E	4q32.3	1984	TTTACAAAGA	3.46E-03
	↑	42	18	RPS8	Ribosomal protein S8	1p34.1-p32	671/674	TAATAAAGGT	4.61E-03
	↑	18	4	SCG5	Secretory granule, neuroendocrine protein 1 (7B2 protein)	15q13-q14	1168	AATGCAAGCA	5.22E-03
	↓	9	22	RPL13	Ribosomal protein L13	17p11.2	504	CCCGTCCGGA	6.19E-03
	↓	4	14	RPS27A	Ribosomal protein S27a	2p16	522	AACTAAAAAA	8.64E-03

Transport	↑	17	2	FXYD6	FXYD domain containing ion transport regulator 6	11q23.3	1344	GGCTCTGGGA	8.77E-04
	↑	43	18	ATP1A1	ATPase, Na+/K+ transporting, alpha 1 polypeptide	1p21	3479	TAGCTCTATG	3.76E-03
	↓	9	23	GABARAPL2	GABA(A) receptor-associated protein-like 2	16q22.3-q24.1	865	TTGGACTGAG	4.45E-03
	↑	20	5	NAPA	N-ethylmaleimide-sensitive factor attachment protein, alpha	19q13.32	1028	TAGCCGCTGA	4.92E-03
	↑	18	4	ATP1B1	ATPase, Na+/K+ transporting, beta 1 polypeptide	1q24	2068	TTCTAACATA	5.22E-03
	↓	8	21	ATP1B2	ATPase, Na+/K+ transporting, beta 2 polypeptide	17p13.1		GTCTCTCTCT	5.31E-03
	↓	3	13	ATP2B4	ATPase, Ca++ transporting, plasma membrane 4	1q32.1	8958	CCCACTTGTA	5.74E-03
	↓	15	30	VAMP2	Synaptobrevin 2	17p13.1	846	CTCCCTCTGC	6.79E-03
	↓	2	11	STX1B2	Syntaxin 1B2	16p12-p11	2233	TCCTGTCCCC	6.92E-03
	↑	15	3	TIMM17A	Translocase of inner mitochondrial membrane 17 homolog A (yeast)	1q32.1	1254	GAGAGTGTAC	8.03E-03
Immune response	↓	3	17	HLA-A	Major histocompatibility complex, class I, A	6p21.3	1362	GTGCACTGAG	5.47E-04
**** Anti-apoptosis*	↓	1	11	IFITM3	Interferon induced transmembrane protein 3 (1-8U)	11p15.5	499	ACCTGTATCC	1.70E-03
	↑	52	23	IFI6	*** Interferon, alpha-inducible protein (clone IFI-6-16)	1p35	696	CGCCGACGAT	3.13E-03
	↓	112	140	CLU	Clusterin	8p21-p12	1630	CAACTAATTC	4.69E-03
Cytokine and chemokine mediated signaling pathway	↓	2	18	CCL15	Chemokine (C-C motif) ligand 14	17q11.2	482	CTCAAAAAAA	8.67E-05
Ion Transport	↓	13	50	*ATP5E*	ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit	20q13.32	313	GGAAAAAAAA	0
Regulation of gene expression, epigenetic	↑	19	4	CSDE1	cold shock domain containing E1, RNA-binding	1p22	3535	CAAATGAGGA	2.91E-03
Biological_process unknown	↓	1	32	*MALAT1*	Metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA)	11q13.1		ACGCAGGGAG	0
	↑	31	6	ZER1	zer-1 homolog (C. elegans)	9q34.11	4143	CGGCGCTCCC	6.67E-05
	↓	32	63	HSPC132	Hypothetical protein HSPC132	12q24.31	229	GCAAAGAAAA	1.30E-04
	↑	74	32	MEG3	Maternally expressed 3	14q32	3263	TGGGAAGTGG	2.83E-04
	↓	0	9	FAM107A	family with sequence similarity 107, member A	3p21.1	1665	ATTGCCAATC	1.25E-03
	↓	4	17	STAC2	SH3 and cysteine rich domain 2	17q12	3149	ATGGGTCTGG	1.55E-03
	↓	3	15	CMTM4	Chemokine-like factor super family 4	16q22.1	3381	AAGGAACTTG	1.79E-03
	↓	0	8	C9orf90	Chromosome 9 open reading frame 90	9q34.11	2226/2229	ATGAAACCCC	2.56E-03
	↑	18	4	FNDC4	Fibronectin type III domain containing 4	2p23.3	1203	GAGCCTCAGG	5.22E-03
	↓	0	7	CCDC120	JM11 protein	Xp11.23	3638	CCCGGCTAAT	5.28E-03
	↓	1	9	NT5C3L	Hypothetical protein MGC20781	17q21.2	1309	GGAAGTTCAA	6.82E-03
	↓	4	14	CUTA	Brain acetylcholinesterase putative membrane anchor	6pter-p21.31		AAGATCCCCG	8.64E-03
	↓	6	17	PIGY	Hypothetical protein MGC14156	4q22.1	1283	TTGACACTTT	8.66E-03

3.3) Genes com expressão diferencial sugerida por SAGE

Desde os primórdios da pesquisa de cunho molecular na SCZ, atribui-se as causas da SCZ principalmente a uma disfunção dopaminérgica, como detalhado na Introdução desta tese. Entretanto, recentes pesquisas mostram outros fatores como a disfunção dos oligodendrócitos, o possível envolvimento de diversos genes de pequeno efeito e fatores ambientais interferem, de fato, no estabelecimento e desenvolvimento da SCZ.

Os dados obtidos a partir de análises das SAGE *tags* geradas do sequenciamento dos clones das bibliotecas SCZ-lib e CTRL-lib confirmam dados previamente gerados.

3.3.1) Alterações na função sináptica:

As primeiras análises de expressão gênica em larga-escala de tecidos de pacientes com SCZ foram feitas no início desta década usando cDNA *microarrays* [2,3,4,5]. Dentre os achados mais relevantes destes estudos estavam a expressão diferencial de genes, cujos produtos exercem função sináptica, e têm sido alvo de pesquisas em SCZ ja há vários anos, incluindo-se o desbalanço da atividade dopaminérgica [revisado em 6]. A alteração no metabolismo sináptico na SCZ pode ser fruto de uma disfunção do neurodesenvolvimento[7] o que causa déficit de memória e mau funcionamento do circuito hipocampal[8].

Nossas análises do transcriptoma do córtex pré-frontal de pacientes com SCZ usando SAGE, reforçam a importância da função sináptica nesta doença. Nossos dados apontam para alguns genes já previamente descritos por outros grupos como envolvidos com a SCZ, tais como STX1B2[4], CALM[4], YWHAH[5] e

[2] Mirnics K, Middleton FA, Marquez A, Lewis DA, Levitt P. Molecular characterization of schizophrenia viewed by microarray analysis of gene expression in prefrontal cortex. Neuron. 2000 Oct;28(1):53-67.
[3] Hakak Y, Walker JR, Li C, Wong WH, Davis KL, Buxbaum JD, Haroutunian V, Fienberg AA. Genome-wide expression analysis reveals dysregulation of myelination-related genes in chronic schizophrenia. Proc Natl Acad Sci U S A. 2001 Apr 10;98(8):4746-51.
[4] Vawter MP, Barrett T, Cheadle C, Sokolov BP, Wood WH 3rd, Donovan DM, Webster M, Freed WJ, Becker KG. Application of cDNA microarrays to examine gene expression differences in schizophrenia. Brain Res Bull. 2001 Jul 15;55(5):641-50.
[5] Middleton FA, Mirnics K, Pierri JN, Lewis DA, Levitt P. Gene expression profiling reveals alterations of specific metabolic pathways in schizophrenia. J Neurosci. 2002 Apr 1;22(7):2718-29.
[6] Owen MJ, O'Donovan MC, Harrison PJ. Schizophrenia: a genetic disorder of the synapse? BMJ 2005;330: 158 – 159.
[7] Raedler TJ, Knable MB, Weinberger DR. Schizophrenia as a developmental disorder of the cerebral cortex. Curr Opin Neurobiol. 1998 8(1):157-161.
[8] Ben-Shachar D, Laifenfeld D. Mitochondria, synaptic plasticity, and schizophrenia. Int Rev Neurobiol. 2004 59:273-296.

VAMP2[9]. Além disto, identificamos alterações quantitativas na expressão de outros dois genes envolvidos com função sináptica, SYNPO e NRGN, ainda não descritos por outros grupos.

Em nossas análises do proteoma de tecido cerebral de pacientes com SCZ, como descreveremos nos capítulos seguintes, novamente observamos alterações na expressão de YWHAH e CALM, reforçando os achados prévios e os nossos dados de SAGE, sinalizando para a robustez destes potenciais marcadores.

A expressão diferencial destes genes e proteínas acima descritos pode gerar distúrbio sináptico, que pode levar a uma série de alterações no circuito neuronal.

3.3.2) Alterações no metabolismo energético:

A primeira vez que a relação entre metabolismo energético e SCZ foi descrita foi em 1954 por Takahashi[10], que observou que a glicólise aeróbica era menor em cérebros SCZ *post mortem.* A partir de então diversos estudos em diferentes áreas do conhecimento passaram a buscar a comprovação do distúrbio energético celular, tentando compreender suas raízes. Estudos de expressão gênica[5,11,12] e protéica[13,14] em larga escala têm comprovado extensivamente este postulado, identificando genes e proteínas que possam ser gatilhos para esta disfunção na SCZ. Ainda, a proximidade do metabolismo energético com a plasticidade neuronal e sinapses[8] além do dano oxidativo previamente observado[revisado em 15], sugerem que o metabolismo energético diferencial em cérebros de pacientes com SCZ é um componente importante nesta patogenia.

[9] Halim ND, Weickert CS, McClintock BW, Hyde TM, Weinberger DR, Kleinman JE, Lipska BK.Presynaptic proteins in the prefrontal cortex of patients with schizophrenia and rats with abnormal prefrontal development.Mol Psychiatry. 2003 Sep;8(9):797-810.
[10] Takahashi Y. An enzymological study on brain tissue of schizophrenic patients. Carbohydrate metabolism. Folia Psychiatrica Neurol. Japonica 1954;7, 214–237.
[11] Prabakaran S, Swatton JE, Ryan MM, Huffaker SJ, Huang JT, Griffin JL, Wayland M, Freeman T, Dudbridge F, Lilley KS, Karp NA, Hester S, Tkachev D, Mimmack ML, Yolken RH, Webster MJ, Torrey EF, Bahn S. Mitochondrial dysfunction in schizophrenia: evidence for compromised brain metabolism and oxidative stress. Mol Psychiatry. 2004 Jul;9(7):684-97, 643.
[12] Glatt SJ, Everall IP, Kremen WS, Corbeil J, Sásik R, Khanlou N, Han M, Liew CC, Tsuang MT.Comparative gene expression analysis of blood and brain provides concurrent validation of SELENBP1 up-regulation in schizophrenia. Proc Natl Acad Sci U S A. 2005 Oct 25;102(43):15533-8
[13] Karry R, Klein E, Ben-Shachar D. Mitochondrial complex I subunits expression is altered in schizophrenia: a postmortem study.Biol Psychiatry. 2004;55(7):676-684
[14] Clark D, Dedova I, Cordwell S, Matsumoto I. A proteome analysis of the anterior cingulate cortex gray matter in schizophrenia. Mol Psychiatry. 2006;11:459-470.
[15] Yao JK, Reddy RD, van Kammen DP. Oxidative damage and schizophrenia: an overview of the evidence and its therapeutic implications. CNS Drugs. 2001;15(4):287-310.

Em nossas análises por SAGE do transcriptoma do córtex pré-frontal de pacientes com SCZ confirmamos a expressão diferencial de genes cujos produtos têm função no metabolismo energético como os genes MDH1[5], ATP5A1[5], TIMM17A[5], além da identificação de alguns genes encontrados diferencialmente expressos exclusivamente por nossa análise como NDUFA1, PDXK e DLD.

Em nossas análises do proteoma de tecido cerebral de pacientes com SCZ, encontramos alterações na expressão das proteínas similares a NDUFA1 e ALDOA como encontradas por SAGE além de outras proteínas desta via, que novamente reforçam os achados prévios e nossos achados das bibliotecas SAGE.

3.3.3) Alteração no metabolismo de oligodendrócitos:

Dentre as suas várias funções, tais como sinalização trófica com outros neurônios, síntese de fatores de crescimento, sustentação física e química dos neurônios e propagação de sinais, talvez a principal atividade dos oligodendrócitos seja a formação da bainha de mielina ao redor dos axônios neuronais no SNC, o que permite a efetiva propagação dos impulsos elétricos[16].

A maioria dos estudos de expressão gênica global de cérebros de pacientes com SCZ feitos por cDNA *microarrays*[3,17,18,19,20] revelaram uma disfunção no metabolismo dos oligodendrócitos, trazendo uma nova perspectiva para os estudos moleculares em SCZ, o que de certo modo descentralizou o foco das pesquisas que muito estudavam a disfunção dopaminérgica, hipótese mais fundamentada da SCZ[21]. Alguns genes descritos por estes estudos globais foram validados por outros grupos[22,23], fundamentando ainda mais a recente hipótese da disfunção dos oligodendrócitos.

[16] Du Y, Dreyfus CF. Oligodendrocytes as providers of growth factors. J Neurosci Res. 2002 Jun 15;68(6):647-54.
[17] Tkachev D, Mimmack ML, Ryan MM, Wayland M, Freeman T, Jones PB, Starkey M, Webster MJ, Yolken RH, Bahn S. Oligodendrocyte dysfunction in schizophrenia and bipolar disorder. Lancet. 2003 Sep 6;362(9386):798-805..
[18] Aston C, Jiang L, Sokolov BP. Microarray analysis of postmortem temporal cortex from patients with schizophrenia. J Neurosci Res. 2004 Sep 15;77(6):858-66.
[19] Katsel P, Davis KL, Haroutunian V. Variations in myelin and oligodendrocyte-related gene expression across multiple brain regions in schizophrenia: a gene ontology study. Schizophr Res. 2005 Nov 15;79(2-3):157-73.
[20] Arion D, Unger T, Lewis DA, Levitt P, Mirnics K. Molecular evidence for increased expression of genes related to immune and chaperone function in the prefrontal cortex in schizophrenia. Biol Psychiatry. 2007 Oct 1;62(7):711-21.
[21] Segal D, Koschnick JR, Slegers LH, Hof PR. Oligodendrocyte pathophysiology: a new view of schizophrenia.Int J Neuropsychopharmacol. 2007 Aug;10(4):503-11.
[22] Dracheva S, Davis KL, Chin B, Woo DA, Schmeidler J, Haroutunian V. Myelin-associated mRNA and protein expression deficits in the anterior cingulate cortex and hippocampus in elderly schizophrenia patients. Neurobiol Dis. 2006; 21(3):531-540.

Em nossas análises por SAGE do transcriptoma do córtex pré-frontal de pacientes com SCZ identificamos genes previamente descritos em SCZ cujos produtos estão envolvidos com o metabolismo de oligodendrócitos como os genes PMP2[22], MAG[3,17,22,23] e MOBP[24]. Ainda, encontramos alterações em alguns genes desta via, nunca antes relacionados como potenciais marcadores na SCZ, incluindo SMPD1 e SMPD4, apesar do metabolismo de esfingomielina já ter sido descrito previamente[25,26] e comprovamos a expressão gênica diferencial de MBP que, apesar de toda a pesquisa acerca deste gene, nenhum outro estudo de expressão global de RNA havia comprovado consistentemente sua expressão diferencial. Cabe notar que nossos dados de proteoma desta mesma região, apontaram mais uma vez para a alteração deste gene (vide capítulos 4, 5 e 6)

As vias até aqui descritas são discutidas em maior detalhe no Capítulo 2 desta tese.

3.3.4) Alteração na expressão de genes relacionados a homeostase de cálcio:

Apesar da idéia que se tem sobre o envolvimento da homeostase do íon cálcio (Ca^{+2}) na patogênese da SCZ, não se sabe ao certo como este influi na doença. Ca^{+2} desempenha um papel central em diversos aspectos da função celular, incluindo diversos mecanismos de sinalização e até mesmo a conformação tridimensional de proteínas[27]. Por sua importância na função de receptores dopaminérgicos o Ca^{+2} é apontado por alguns pesquisadores como metabólito central da hipótese dopaminérgica da SCZ[28]. Sabe-se ainda que o aumento da atividade de fosfolipases A2 (PLA2) dependentes de Ca^{+2}, que pode variar de acordo com as quantidades de Ca^{+2} disponíveis[29], podendo acelerar o *turnover* de fosfolípides de membrana e reduzir a atividade dopaminérgica

[23] McCullumsmith RE, Gupta D, Beneyto M, Kreger E, Haroutunian V, Davis KL, Meador-Woodruff JH. Expression of transcripts for myelination-related genes in the anterior cingulate cortex in schizophrenia. Schizophr Res. 2007; 90(1-3):15-27.
[24] Aston C, Jiang L, Sokolov BP. Transcriptional profiling reveals evidence for signaling and oligodendroglial abnormalities in the temporal cortex from patients with major depressive disorder. Mol Psychiatry. 2005 Mar;10(3):309-22.
[25] Schmitt A, Wilczek K, Blennow K, Maras A, Jatzko A, Petroianu G, Braus DF, Gattaz WF. Altered thalamic membrane phospholipids in schizophrenia: a postmortem study. Biol Psychiatry. 2004 Jul 1;56(1):41-5.
[26] Fonteh AN, Harrington RJ, Huhmer AF, Biringer RG, Riggins JN, Harrington MG. Identification of disease markers in human cerebrospinal fluid using lipidomic and proteomic methods. Dis Markers. 2006;22(1-2):39-64.
[27] Clapham DE. Calcium signaling. Cell. 2007 Dec 14;131(6):1047-58.
[28] Bergson C, Levenson R, Goldman-Rakic PS, Lidow MS. Dopamine receptor-interacting proteins: the Ca(2+) connection in dopamine signaling. Trends Pharmacol Sci. 2003; 24(9):486-492.
[29] Gattaz WF, Hubner CV, Nevalainen TJ, Thuren T, Kinnunen PK. Increased serum phospholipase A2 activity in schizophrenia: a replication study. Biol Psychiatry. 1990; 28(6):495-501.

cerebral[30]. Além disso, um estudo de associação genética em SCZ apontou um canal de potássio ativado por cálcio como uma molécula importante na patogênese da SCZ[31].

Em nossas análises por SAGE identificamos e expressão diferencial de genes codificadores de proteínas envolvidas com o metabolismo de Ca^{+2}, previamente associados com a SCZ, tais como CALM1[4], S100B[32,33] além outros genes inéditos desta mesma via, tais como CLSTN1 e CAMK2B.

Em nossos estudos de proteoma, como será detalhado nos próximos capítulos desta tese, também observamos a alteração na expressão de diversas proteínas envolvidas com a homeostase do Ca^{+2}, dentre as quais algumas também encontradas por SAGE como CALM e PPP3CA (calcineurina), o que tanto reforça nossos achados quanto sugere real envolvimento da homeostase deste íon na SCZ.

3.4) Validação da expressão gênica diferencial em amostras de pacientes com esquizofrenia:

Análises de *pools* de amostras, como feito por nós nos estudos de SAGE tem como intuito prover uma verificação conjunta da expressão gênica que permita diluir diferenças individuais de expressão gênica e buscando evidenciar as alterações comuns à maioria das amostras, já que estas poderiam ser importantes na determinação do fenótipo. Entretanto estas diferenças comuns descobertas nas análises de um *pool* devem ser posteriormente validadas em amostras individuais (incluindo aquelas que compuseram o *pool* e também em outras) para a validação da expressão gênica diferencial. Evidentemente não descartamos a importância das diferenças individuais, as quais poderiam inclusive ter uma importância na determinação dos endofenótipos. No entanto, o intuito deste trabalho é buscar alterações mais robustas, que possam

[30] Gattaz WF, Brunner J. Phospholipase A2 and the hypofrontality hypothesis of schizophrenia. Prostaglandins Leukot Essent Fatty Acids. 1996; 55(1-2):109-113.
[31] Chandy KG, Fantino E, Wittekindt O, Kalman K, Tong LL, Ho TH, Gutman GA, Crocq MA, Ganguli R, Nimgaonkar V, Morris-Rosendahl DJ, Gargus JJ. Isolation of a novel potassium channel gene hSKCa3 containing a polymorphic CAG repeat: a candidate for schizophrenia and bipolar disorder? Mol Psychiatry. 1998 Jan;3(1):32-7.
[32] Lara DR, Gama CS, Belmonte-de-Abreu P, Portela LV, Gonçalves CA, Fonseca M, Hauck S, Souza DO.Increased serum S100B protein in schizophrenia: a study in medication-free patients.J Psychiatr Res. 2001 Jan-Feb;35(1):11-4.
[33] Rothermundt M, Missler U, Arolt V, Peters M, Leadbeater J, Wiesmann M, Rudolf S, Wandinger KP, Kirchner H.Increased S100B blood levels in unmedicated and treated schizophrenic patients are correlated with negative symptomatology.Mol Psychiatry. 2001 Jul;6(4):445-9.

caracterizar marcadores consistentes e potenciais alvos farmacológicos para os pacientes portadores de SCZ, de um modo geral.

Uma parte importante dos dados gerados por SAGE inclui a observação da expressão diferencial de diversos genes nunca antes relacionados à SCZ. Deste modo, selecionamos alguns destes para uma análise nas amostras de pacientes individuais, por PCR quantitativa em tempo real (qPCR), buscando observar qual o percentual de pacientes que apresentava estes genes regulados, conforme indicado por SAGE. Os genes selecionados para esta análise foram escolhidos com base em dois critérios: 1) uma alta diferença de expressão entre os *pools* de controle e SCZ e 2) o ineditismo de sua correlação com a SCZ. Os genes selecionados foram MALAT1, KIF1C, ATP5E, e CSRP1.

A expressão destes genes foi avaliada individualmente em cerca de 10 amostras CTRL e 9 amostras SCZ (Tabela 1). Para realizarmos os cálculos de expressão diferencial, analisamos o nível de expressão de cada gene, individualmente, em todas as amostras disponíveis. A expressão foi normalizada por um controle endógeno. A partir daí, calculamos a expressão dos genes alvo em cada uma das amostras SCZ, determinando a variação de cada amostra em comparação com o valor de expressão média observada nas amostras controle (expressão nos controles = 1).

A seguir, discutiremos em maior detalhe estes potenciais novos marcadores da SCZ, e apresentaremos os resultados de sua validação em amostras individuais.

*** MALAT1** - *Metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA)* - Freqüência das *tags* observada por SAGE = 32/50,000 CTRL versus 1/50,000 SCZ (expressão reduzida em 32x em SCZ - P=0).

Este gene está numa região do genoma possivelmente relacionada à SCZ (cromossomo 11q) [34,35], entretanto, não existe nada na literatura acerca de sua

[34] Williams NM, Rees MI, Holmans P, Norton N, Cardno AG, Jones LA, Murphy KC, Sanders RD, McCarthy G, Gray MY, Fenton I, McGuffin P, Owen MJ. A two-stage genome scan for schizophrenia susceptibility genes in 196 affected sibling pairs. Hum Mol Genet. 1999 Sep;8(9):1729-39.

[35] Lewis CM, Levinson DF, Wise LH, DeLisi LE, Straub RE, Hovatta I, Williams NM, Schwab SG, Pulver AE, Faraone SV, Brzustowicz LM, Kaufmann CA, Garver DL, Gurling HM, Lindholm E, Coon H, Moises HW, Byerley W, Shaw SH, Mesen A, Sherrington R, O'Neill

função no SNC, nem sequer qualquer descrição de uma possível correlação com SCZ. Cabe notar que, como um RNA não codificador, este gene deve ter um papel regulatório sobre outros transcritos. A única descrição deste gene se refere à sua expressão significativamente associada à metástase em pacientes com câncer de pulmão[36]. Devido à sua significativa diferença de expressão, seu mapeamento e seu ineditismo, incluímos este gene no estudo.

Como apresentado no Gráfico 1, vemos que não foi possível detectar diferenças significativas na expressão de *MALAT1* entre as amostras do grupo SCZ ou do grupo controle. A diferença de expressão observada entre SAGE e qPCR pode ser explicada por diversos motivos tais como mapeamento ambíguo das *tags*, presença de isoformas de *splicing,* poli-adenilação alternativa, diferenças entre as técnicas empregadas, ou ausência real de expressão diferencial. No entanto, diante dos promissores achados sugeridos por SAGE, acreditamos subsequentes validações em outras amostras ainda sejam justificáveis.

FA, Walsh D, Kendler KS, Ekelund J, Paunio T, Lonnqvist J, Peltonen L, O'Donovan MC, Owen MJ, Wildenauer DB, Maier W, Nestadt G, Blouin JL, Antonarakis SE, Mowry BJ, Silverman JM, Crowe RR, Cloninger CR, Tsuang MT, Malaspina D, Harkavy-Friedman JM, Svrakic DM, Bassett AS, Holcomb J, Kalsi G, McQuillin A, Brynjolfson J, Sigmundsson T, Petursson H, Jazin E, Zoega T, Helgason T. Genome scan meta-analysis of schizophrenia and bipolar disorder, part II: Schizophrenia. Am J Hum Genet. 2003 Jul;73(1):34-48.

[36] Ji P, Diederichs S, Wang W, Boing S, Metzger R, Schneider PM, Tidow N, Brandt B, Buerger H, Bulk E, Thomas M, Berdel WE, Serve H, Muller-Tidow C. MALAT-1, a novel noncoding RNA, and thymosin beta4 predict metastasis and survival in early-stage non-small cell lung cancer. Oncogene. 2003 Sep 11;22(39):8031-41.

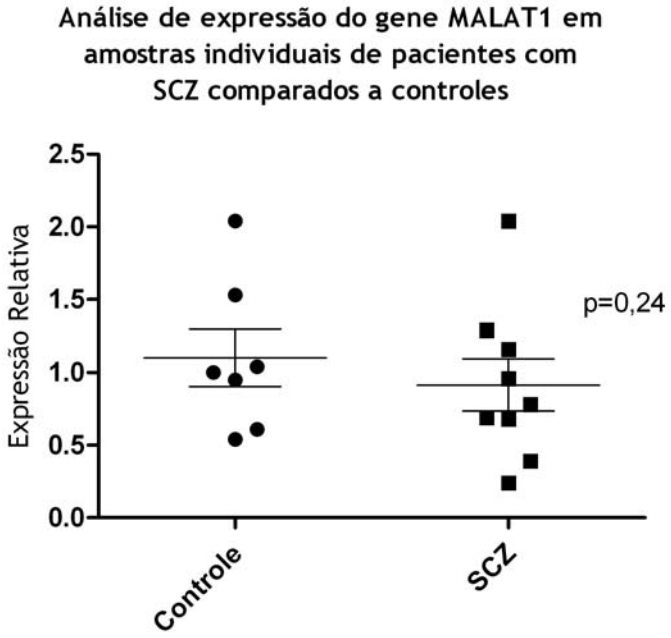

Gráfico 1: Dados da expressão gênica individual de MALAT1 em córtex pré-frontal de pacientes com SCZ e controles.

*** KIF1C -** *Kinesin family member 1C* - Freqüência das *tags* observada por SAGE = 22/50,000 CTRL versus 3/50,000 SCZ (expressão reduzida em 7,33x em SCZ - P=2,33E-05).

Localizado no braço curto do cromossomo 17, este gene é um novo membro da família dos genes KIF (kinesin family) cuja atividade inicialmente identificada envolvia participação no transporte de vesículas do complexo de Golgi ao retículo endoplasmático (RE), assim como outros genes desta mesma família[37]. Em seguida, alguns trabalhos revelaram a importância deste gene em macrófagos de linhagens de ratos resistentes ao fator letal provindo do Antrax[38]. Algumas linhagens de ratos eram resistentes por apresentarem modificações neste gene. Porém, recentes pesquisas revelaram que o produto deste gene é dispensável em relação à organização dos microtúbulos no transporte de

[37] Dorner C, Ciossek T, Muller S, Moller PH, Ullrich A, Lammers R. Characterization of KIF1C, a new kinesin-like protein involved in vesicle transport from the Golgi apparatus to the endoplasmic reticulum. J Biol Chem. 1998 Aug 7;273(32):20267-75.
[38] Watters JW, Dewar K, Lehoczky J, Boyartchuk V, Dietrich WF. Kif1C, a kinesin-like motor protein, mediates mouse macrophage resistance to anthrax lethal factor. Curr Biol. 2001 Oct 2;11(19):1503-11.

vesículas Golgi – RE[39]. Logo, sua função ainda não é totalmente compreendida. Porém, um dado importante: em trabalho publicado por Dorner e colaboradores em 1999[40] aponta a interação de KIF1C com produtos da família YWHA. Um dos genes desta da família YWHAH foi por nós apontado como um dos mais diferencialmente expressos em SCZ.

Como apresentado no Gráfico 2, observamos que houve uma tendência geral de redução de expressão de KIFC1 quando as amostras do grupo SCZ foram comparadas aos controles (p=0,05). Consideramos portanto que KIF1C é um promissor biomarcador, que deve ser avaliado em um número maior de amostras.

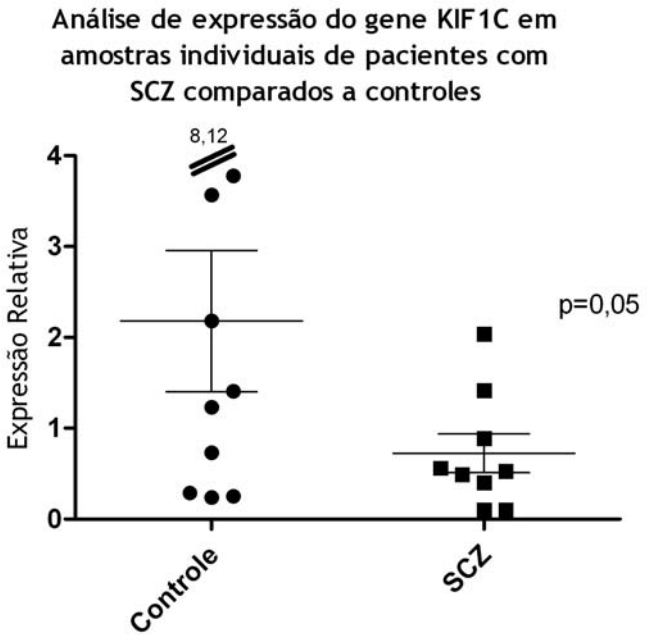

Gráfico 2: Dados da expressão gênica individual de KIF1C em córtex pré-frontal de pacientes com SCZ e controles.

*** ATP5E** - *ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit* - Freqüência das *tags* observada por SAGE = 50/50,000 CTRL versus 13/50,000 SCZ (expressão reduzida em 3,85x em SCZ - P=0).

[39] Nakajima K, Takei Y, Tanaka Y, Nakagawa T, Nakata T, Noda Y, Setou M, Hirokawa N. Molecular motor KIF1C is not essential for mouse survival and motor-dependent retrograde Golgi apparatus-to-endoplasmic reticulum transport. Mol Cell Biol. 2002 Feb;22(3):866-73.
[40] Dorner C, Ullrich A, Haring HU, Lammers R. The kinesin-like motor protein KIF1C occurs in intact cells as a dimer and associates with proteins of the 14-3-3 family. J Biol Chem. 1999 Nov 19;274(47):33654-60.

Este gene encontra-se no braço longo do cromossomo 20, uma outra região do genoma possivelmente relacionada com a SCZ[34,35]. Paulson e colaboradores em 2003 e 2004 publicaram trabalhos onde propõem um modelo animal (rato) para SCZ utilizando uma substância denominada MK-801, um antagonista de glutamato, que se liga a receptores NMDA, que na SCZ pode ser a causa dos sintomas negativos. Cabe notar que neste modelo animal, o gene ATP5E teve a sua expressão alterada em tálamo e córtex frontal dos ratos tratados com MK-801. O produto da ATP5E é uma subunidade da ATP sintase, complexo protéico envolvido com a produção de ATP na célula. Muitos achados em relação a alterações em proteínas mitocondriais mostram que genes relacionados à mitocôndria podem ter relação íntima com a SCZ. A função nervosa e sistemas de neurotransmissão são intimamente ligados ao metabolismo energético desempenhado pela mitocôndria[41]. A primeira vez que foi descrição da relação entre metabolismo mitocondrial e SCZ foi feita em 1954 por Takahashi, que observou que a glicólise aeróbica era menor em cérebros SCZ *post mortem*, mas não se sabe se isso é causa ou conseqüência da doença. É sabido que antipsicóticos podem gerar modificações na expressão de genes mitocondriais[8], o que pode justificar (ou não) os achados de Takahashi. Outros achados: Prince e colaboradores em 1999 apotaram mudanças no metabolismo de energia em SCZ em regiões específicas dos núcleos da base de cérebros SCZ. Por PET (Positron Emission Tomography - Tomografia por Emissão de Pósitrons), observou-se em cérebros SCZ queda no metabolismo oxidativo em cortex frontal[42]. Estudos clínicos demonstraram menor taxa metabólica mitocondrial em áreas corticais de cérebros SCZ[43]. No entanto, uma análise destes dados requer a cuidadosa avaliação de idade, uso de álcool e tabaco, já que estes fatores, entre outros, podem levar a uma redução do metabolismo cerebral[8].

[41] Mulcrone J, Whatley SA, Ferrier IN, Marchbanks RM. A study of altered gene expression in frontal cortex from schizophrenic patients using differential screening. Schizophr Res. 1995 Feb;14(3):203-13.
[42] Cohen RM, Semple WE, Gross M, Nordahl TE, Holcomb HH, Dowling S, Pickar D. The effect of neuroleptics on dysfunction in a prefrontal substrate of sustained attention in schizophrenia. Life Sci. 1988;43, 1138-1150
[43] Cavelier L, Jazin EE, Eriksson I, Prince J, Båve U, Oreland L and Gyllensten U. Decreased Cytochrome-c Oxidase Activity and Lack of Age-Related Accumulation of Mitochondrial DNA Deletions in the Brains of Schizophrenics. Genomics. 1995; 29(1), 217-224.

A discussão acerca do metabolismo do SNC de pacientes SCZ relacionado ao metabolismo mitocondrial e dopamina é algo bastante difundido, com muitos dados já conhecidos. No entanto a completa compreensão de todos os processos envolvidos ainda não foi atingida. Por isso, à medida que descobrimos a alteração de novos genes relacionados ao metabolismo mitocondrial contribuímos para a construção de um quadro mais completo deste processo. Sabendo-se que dopamina, metabolismo mitocondrial e SCZ estão correlacionados, tem-se o seguinte raciocínio: A SCZ parece estar relacionada a uma disfunção no metabolismo da dopamina que em altas concetrações no cérebro pode causar dano pré e pós-sináptico[44] e, consequentemente, morte celular. Isso porque altas concentrações de dopamina quando metabolizadas geram radicais livres, ou seja, estresse oxidativo logo, descréscimo no metabolismo mitocondrial[12]. Deste modo, uma das causas (ou consequências?) da SCZ poderia ser a inabilidade do cérebro em manter níveis normais de ATP causadas por alterações no metabolismo mitocondrial devido a estes danos oxidativos. Estudos mostram que quando há tratamento no cérebro com derivados neurotóxicos da dopamina, a concentração de ATP cai[45,46]. Outros experimentos mostraram que quando se administra em ratos drogas que induzem a produção de dopamina, o complexo I da mitocôndria reduz sua atividade, pois o metabolismo da dopamina produz metabólitos tóxicos, que em grandes quantidades podem inibir fortemente o metabolismo mitocondrial[47]. Estes dois estudos podem explicar a baixa de ATP em cérebros SCZ, devido a uma maior metabolização da dopamina. Visto tal discussão, qualquer alvo molecular relacionado ao metabolismo mitocondrial por nós encontrado deve ser tido um alvo em potencial como causa ou consequência da SCZ.

[44] Carlsson A. Antipsychotics drugs, neurotransmitters, and schizophrenia. Am J Psychiatry 1978;135:165-173.
[45] Chan P, Di Monte DA, Luo JJ, DeLanney LE, Irwin I, Langston JW. Rapid ATP loss caused by methamphetamine in the mouse striatum: relationship between energy impairment and dopaminergic neurotoxicity.J Neurochem. 1994 Jun;62(6):2484-7.
[46] Royland JE, Delfani K, Langston JW, Janson AM, Di Monte DA.7-Nitroindazole prevents 1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine-induced ATP loss in the mouse striatum.Brain Res. 1999 Aug 21;839(1):41-8.
[47] Ben-Shachar D, Zuk R, Gazawi H, Ljubuncic P. Dopamine toxicity involves mitochondrial complex I inhibition: implications to dopamine-related neuropsychiatric disorders. Biochem Pharmacol. 2004;67(10):1965-1974.

Como apresentado no Gráfico 3, observamos uma importante redução na expressão de ATP5E em SCZ quando comparado às amostras controle. As diferenças apresentadas são estatisticamente significativas (p=0,04).

Interessante observar o comportamento heterogêneo da expressão deste gene entre os controles, enquanto que nos pacientes com SCZ a variação de expressão é menor, o não reduz o potencial deste gene como potencial biomarcador em SCZ.

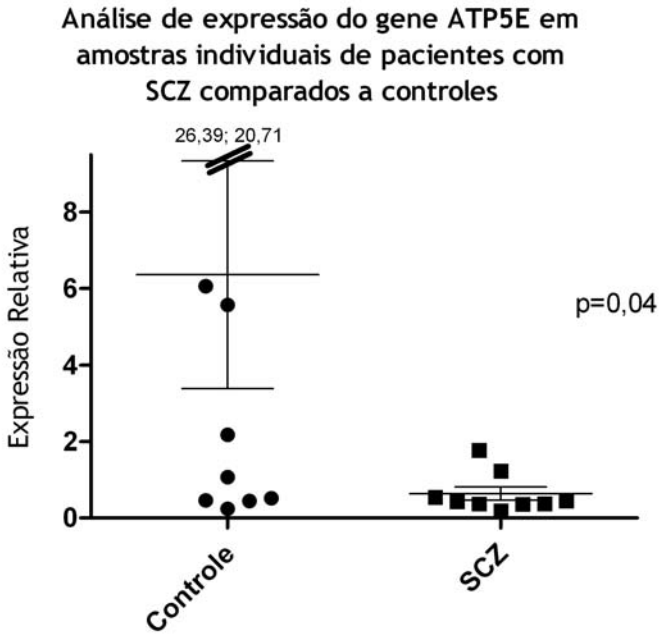

Gráfico 3: Dados da expressão gênica individual de ATP5E em córtex pré-frontal de pacientes com SCZ e controles.

*** CSRP1 -** Cysteine and glycine-rich protein 1 - Freqüência das *tags* observada por SAGE = 40/50,000 CTRL versus 13/50,000 SCZ (expressão reduzida em 3,08x em SCZ - P=0).

Este gene está no braço longo do cromossomo 1, uma grande região genômica, contendo alguns outros genes possivelmente envolvidos com a SCZ[34,35]. A CSRP1 pertence à família das *cystein rich proteins* que tem importante papel regulatório no desenvolvimento e diferenciação celular, organição de do

citoesqueleto e também desenvolvimento neuronal[48,49]. O produto de CSRP1, geralmente localizado no núcleo celular, é regulado na presença de zinco. Este gene ainda não foi relacionado à SCZ.

Como apresentado no Gráfico 4, observamos que a maioria das amostras (6/9) apresentaram uma redução (abaixo de 50%) na expressão de CSRP1 quando comparado à expressão média observada nos controles. Quando o valor médio de todas as amostras SCZ é comparado ao valor médio das amostras controle, observamos em SCZ, uma expressão equivalente a 22% da expressão média no grupo controle. Se observarmos a mediana, esta expressão é de apenas 16%, sendo portanto próximo do valor obtido com SAGE. No entanto, apesar de a diferença não ter sido estatisticamente significativa, acreditamos que CSRP1 pareça ser um marcador promissor para futuros estudos na SCZ.

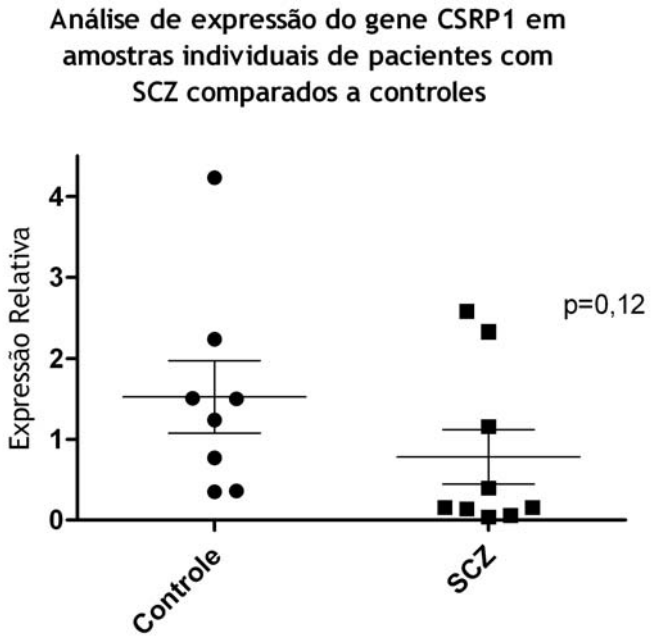

Gráfico 4: Dados da expressão gênica individual de CSRP1 em córtex pré-frontal de pacientes com SCZ e controles.

[48] McLaughlin CR, Tao Q, Abood ME.Isolation and developmental expression of a rat cDNA encoding a cysteine-rich zinc finger protein.Nucleic Acids Res. 1994 Dec 11;22(24):5477-83.
[49] Louis HA, Pino JD, Schmeichel KL, Pomiès P, Beckerle MC.Comparison of three members of the cysteine-rich protein family reveals functional conservation and divergent patterns of gene expression.J Biol Chem. 1997 Oct 24;272(43):27484-91.

4) Conclusão:

A validade da técnica de SAGE no estudo da SCZ pôde ser comprovada pela capacidade de apontar para marcadores consistentemente encontrados em nesta doença, incluindo os genes YWHAH, SYNPO, STX1B2, MDH1, NDUFA1, ATP5A1, ALDOA, CALM1, S100B e MT3, demonstrados em experimentos de *cDNA microarrays* com achados reforçados por nossos dados de proteoma (conforme descrito nos capítulos a seguir). Além disto, não apenas a técnica de SAGE, mas também a estratégia de análise de *pools* mostrou-se viável e adequada, por permitir indicação de promissores novos marcadores moleculares que em sua maioria tiveram a expressão diferencial validada no estudo das amostras individuais. Além dos genes confirmados em nossa breve análise de validação, ainda restam diversos outros marcadores de grande potencial, que ainda devem ser validados, vários dos quais nem sequer enquadram-se em classes funcionais definidas.

Concluímos então que nosso estudo alcançou seu principal objetivo que era fornecer genes que apontam para vias metabólicas importantes e que podem auxiliar na melhor compreensão da SCZ, doença ainda enigmática em muitos aspectos, principalmente devido à influência e interação de diversos fatores endógenos e exógenos.

5) Materiais e Métodos:

5.1) Extração do RNA dos pacientes SCZ e CTRL:

As amostras de córtex pré-frontal dos tecidos cerebrais esquizofrênicos e controles, que estavam armazenadas em –70ºC, foram transferidas para o gelo seco e alíquotas de 50-100mg de tecido por amostra foram usadas na extração após homogenização em 1 ml de TRIzol. A extração do RNA foi feita seguindo as instruções do fabricante do TRIzol (Invitrogen, Carlsbad, EUA).

5.2) Avaliação da qualidade das amostras de RNA de SCZ e CTRL:

Para verificação de degradação das amostras de RNA a serem empregadas nas bibliotecas de SAGE, utilizamos o aparelho Agilent 2100 – Bioanalyzer, segundo protocolo fornecido pelo fabricante (Agilent Technologies - Santa Clara - EUA).

5.3) Construção das Bibliotecas de SAGE:

A biblioteca de SAGE dos pacientes SCZ e controles foram produzidas de acordo com as instruções do fabricante (Invitrogen, Carlsbad, EUA), após modificações. O protocolo consiste basicamente na ligação do mRNA a beads magnéticos que contém cauda poli-A, síntese de uma 1ª fita de DNA complementar ao RNA molde, formando um híbrido dupla-fita DNA-RNA, digestão do RNA molde e síntese da 2ª fita de DNA, complementar a 1a fita, formando um DNA dupla-fita, clivagem com enzima âncora *NlaIII*, ligação dos adaptadores, digestão por *BsmFI*, formando as *tags*, síntese e amplificação das di-*tags*, isolamento das 100pb di-*tags*, clivagem dos adaptadores, concatamerização e seleção dos tamanhos das bibliotecas (geralmente de 300pb a 1Kb). Depois se seguiu a clonagem dos concatâmeros em vetores pZero-1, a transformação de *E. coli* eletrocompetentes, cultivo das bactérias recombinantes, PCR das colônias resultantes para verificação da presença e do tamanho dos insertos. Os produtos de PCR obtidos foram avaliados em géis de agarose corados com brometo de etídio e posteriormente usados para seqüenciamento, como descrito a seguir.

5.4) Sequenciamento dos clones da primeira biblioteca de SAGE:

As bactérias recombinantes foram cultivadas em placas de 96 wells em 100ul de meio de cultura SOB pH 7,00 (2% Triptona, 0,5% Extrato de Levedura, 0,05% NaCl, 1% KCl 250mM, 1% MgCl2 1M, 0,05% Zeocina 100mg/ml) por 15 horas a 37oC.

Após crescidas as bactérias, foi feita a amplificação dos insertos, usando PCR com iniciadores flanqueando o sítio de clonagem do vetor p-Zero-1. Nesta PCR, as bactérias usadas como molde foram coletadas com um repicador de 96 pinos e transferidas para placas de 96 wells contendo 14ul do mix de PCR. O mix

foi submetido à ciclagem térmica (Passo 1: 95oC – 3 minutos; Passo 2: 95oC – 40 segundos; Passo 3: 55oC - 40 segundos; Passo 4: 72oC – 55 segundos; Retornar ao passo 2 por 35 vezes; 72oC por 5 minutos; 4oC infinito).

Alíquotas dos produtos foram testadas em géis de agarose 1% e 1ul da reação de PCR foi coletado e usado como molde para a reação de sequenciamento (10ul volume final), contendo além do primer SP6 (reverso), Big Dye Terminator (version 3). A reação de sequenciamento se deu seguindo os seguintes passos: Passo 1: 95oC – 3 minutos; Passo 2: 95oC – 30 segundos; Passo 3: 55oC - 20 segundos; Passo 4: 60oC – 4 minutos; Retornar ao passo 2 por 35 vezes; 4oC for ever.

Após a reação de sequenciamento, as amostras foram precipitadas com isopropanol 75% e etanol 70%. Após a precipitação, as amostras foram desnaturadas com 10ul formamida e injetadas nos capilares do seqüenciador. O sequenciamento da biblioteca gerada foi feito no LIM-27, num ABI 3100 Genetic Analyzer, da Applied Biosystems em capilares de 36 centimetros e polímero POP6 (Rapid Run).

5.5) Análise inicial dos dados das bibliotecas de SAGE:

O programa utilizado para extração das *tags* dos concatâmeros sequenciados e suas quantificações foi o SAGE2000 Versão 4.5, constituinte do kit I-SAGE (Invitrogen, Carlsbad, EUA) utilizado para a construção das bibliotecas.

Basicamente o SAGE2000 funciona seguindo os seguintes passos: Cria-se um projeto (selecionando a enzima-âncora utilizada e o tamanho das *tags*), importa-se a sequencia crua provinda do sequenciamento da biblioteca (arquivos .seq) e o programa extrai as *tags*. Depois, faz-se o *download* do banco de dados que será usado como referência (ftp do NCBI (National Center of Biological Information - http://www.ncbi.nlm.nih.gov), das pastas Hs (de *Homo sapiens*), *NlaIII*), importa-se este banco de dados no Microsoft Access (que é o programa base usado pelo SAGE2000), as sequencias deste banco são processadas e então os dados do banco de dados e os dados gerados pelo sequenciamento são cruzados, buscando-se cada *tag* sequenciada no banco de dados, quantificando

cada *tag* e comparando a abundância de cada *tag* em diferentes amostras. Para a comparação de diferentes amostras, como feito entre as nossas bibliotecas SCZ-lib e CTRL-lib, o SAGE2000 normaliza o número de *tags* de acordo com um numero selecionado previamente, no nosso caso, 50.000 *tags*.

5.6) Análise da expressão gênica por qPCR:

Oligonucleotídeos iniciadores foram desenhados usando o programa PrimerExpress® (Applied Biosystems, Foster City, CA, EUA). Utilizamos seqüências de cDNA após mascaramento de elementos repetitivos usando RepeatMasker (www.repeatmasker.org)., e buscamos oligos localizados em exons distintos, na tentativa de diminuir contaminações com DNA genômico.

Os ensaios de qPCR foram feitos em duplicatas contendo 1,5 µL de cDNA (cDNA equivalente a 10ng de RNA total), 0,25 picomoles de cada iniciador sense e antisense específicos para cada gene em estudo, 2x SYBR Master Mix (Applied Biosystems, Foster City, CA, USA) contendo dNTPs, enzima Taq e MgCl2 (1,5mM) para um volume final de reação de 15µL. A coleta do sinal de fluorescência foi feita ao final de cada ciclo. Foi feita ainda uma reação com ausência de cDNA (no template control) e controle endógeno normalizador Glucuronidase-beta (GUSB). O programa de ciclagem da reação foi feito com um estágio inicial de 50ºC por 2min, 10 minutos a 95ºC para ativação da Taq DNA polimerase, e a amplificação foi feita em 40 ciclos consistindo cada um em 15 segundos de desnaturação a 95ºC e um minuto a 60ºC para o anelamento e a extensão dos iniciadores.

Após a PCR analisamos as curvas de dissociação que permitem determinar a especificidade dos produtos de PCR obtidos, a partir da análise da temperatura de dissociação (Tm) da dupla fita de DNA formada.

Ciclo de dissociação constituiu de:

- Aquecimento na taxa de 1ºC/segundo, indo de 65 a 95ºC.
- Com subida gradual de temperatura foram coletados os sinais de fluorescência

A análise dos dados foi feita usando o *Sequence Detection Software,* versão 1.3.1 (Applied Biosystems) e os resultados foram exportados para análises

posteriores utilizando o programa Excel. A expressão gênica relativa foi normalizada por um controle endógeno (GUSb) e a expressão diferencial foi calculada no formato padrão usando o método de dCt. Todos os ensaios, bem como todas as amostras usadas por ensaio foram avaliados pelo menos duas vezes, e a média dos valores foi usada para análises estatísticas. Valores de p foram calculados por meio do teste t de Student, usando o programa computacional Biostat.

Capítulo 2:

Biomarcadores de RNA para Esquizofrenia

O capítulo "RNA Biomarkers in Schizophrenia" a seguir é parte do livro "Biomarkers for Psychiatric Disorders" que será publicado na Alemanha pela editora 'Springer', com edição do Prof. Dr. Christoph W. Turck.

RNA Biomarkers in Schizophrenia

Daniel Martins de Souza[1,2] and Emmanuel Dias-Neto[1,3]

1- *Laboratório de Neurociências (LIM-27), Instituto de Psiquiatria, Faculdade de Medicina, Universidade de São Paulo (USP), Brazil.*
2- *Laboratório de Proteômica, Dept. de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Brazil*
3- *Present address: University of Texas, MD Anderson Cancer Center, Houston, TX, USA*

Abstract

The deciphering of the human genome and the advances on transcriptome interrogation approaches, transformed RNA biomarkers in important promises for the understanding and the management of psychiatric diseases. In this chapter we describe the techniques more widely used for gene expression analysis and present the main findings in the search of RNA biomarkers in schizophrenia such as the recurrent observation of alterations in genes that encode proteins involved in pathways related to myelinization, synapses and energy metabolism. We also discuss the main findings resultant from peripheral blood cells studies and present new techniques and new sources of RNA biomarkers for the future of schizophrenia research.

1. Introduction:

The United States Food and Drug Administration (FDA) defines biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic or pathogenic processes or pharmacological responses to a therapeutic intervention" (Chakravarty 2003). The utility of biomarkers in clinical medicine is vast, and includes disease diagnosis, indicators of disease status, or targets to monitor and predict response to therapeutics or disease outcome. In this context, RNAs constitute a central class of markers that can be directly influenced by DNA alterations but may also exert straight influence over protein markers in many diseases.

The primary functional link of RNAs as biomarkers is their obvious roles as intermediates between the DNA that encodes the information, and the proteins that are the ultimate effectors. However, as the RNA complexity continues to be revealed by large-scale transcriptome analysis, new RNA categories continue to emerge bringing new classes of potential markers, including regulatory non-coding RNAs such as miRNAs. The recent discovery of these new RNA molecules will certainly bring dramatic changes in the way we see and understand protein regulation, and will also increase the number of RNA markers available for a number of diseases.

The category of RNA biomarkers gained a dramatic impulse after the deciphering of the human genome, and the diverse projects that contributed to catalogue our transcriptomes. The dynamic nature of the transcriptome reveals the complexity of gene regulation and together with other molecular markers should contribute to the understanding of relevant clinical aspects such as disease mechanisms and reaction to

drugs, pointing to less ambiguous prognosis, and more precise markers for treatment response.

RNA alterations are certainly involved in the biological basis of a number of diseases. In psychiatry, RNA markers are central, and are certainly implicated in diverse facets such as distinct clinical presentations, neuroimaging alterations and endophenotypes. Consistent patterns of abnormalities in proteomics can be a result of alternative mRNA splicing, quantitative fluctuations in mRNA, or translational regulation, which can be determined by non-coding regulatory RNAs. These proteomic alterations can affect diverse disease-related aspects, including neural system alterations that may underlie emotional processing and cognitive control, characteristic of psychiatric diseases. Such abnormalities, present at protein and RNA levels, may constitute valuable disease biomarkers, and may potentially help in the disease diagnosis shedding light on the molecular basis of psychiatric illnesses. Together, genome and transcriptome analysis provided the basis upon which these markers could be investigated and identified by a series of distinct and complementary approaches. In this chapter we will present the most frequently used techniques to interrogate the transcriptome in large-scale and will focus on the most relevant biomarkers that have been revealed in schizophrenia (SCZ), disease uniquely human that affects 1% of the population world wide.

2. The main approaches used for RNA biomarkers discovery

The identification of reliable biomarkers is of utmost relevance for psychiatric diseases. The availability of these markers would be great value for all psychiatric diseases, and of particular importance in SCZ, a complex disease with pathogenesis mechanisms yet

to be defined, despite the various efforts in order to its progressive understanding (Freedman 2003). Besides some macroscopic alterations observed in brain scans of SCZ patients, the disease diagnosis is essentially clinical, with no molecular basis nor reliable biochemical markers identified up to now and an still unpredictable response to treatment (Frances et al. 1991). The complexity of SCZ is a driving force in the search of reliable markers, at DNA, RNA, protein and brain morphometry levels. Besides the recent exploration of the transcriptome in post-genomic era, the search for reliable SCZ markers is still an ongoing process.

At its early days, the search of RNA markers in SCZ was focused in the evaluation of expression of specific genes of interest related to what was known in SCZ. These genes had their cDNA sequenced in samples of patients, such as it has been done in 1986 with the analysis of the coding region of gamma-endorphin gene derived from postmortem brains and pituitary glands of schizophrenic patients (Bovenberg et al. 1986). Nowadays, a number of large-scale techniques allow the interrogation of the entire transcriptome, mainly using hybridization platforms known as gene chips, or cDNA microarrays. The gene arrays available today not only cover the full set of human genes, but also include a large set of non-coding regions and non-coding transcripts, enabling the interrogation of a significant fraction of the whole genome. This comprehensive genome and transcriptome analysis promises a revolution in the comprehension of gene regulation and its effects over human diseases.

The most commonly used techniques for high-throughput transcriptome analysis are cDNA microarrays, Serial Analysis of Gene Expression (SAGE), and the analyses of Expressed Sequence Tags (ESTs). The discrete capabilities and distinct aspects of these different approaches are important to reveal the diversity of gene regulation aspects. These

large-scale methods are absolutely distinct from each other, and not only differ in their throughput and cost, but also in the type of data obtained and in the depth of information provided (Mirnics et al. 2001). These techniques not only allow an analysis of quantitative alterations, an aspect that is the subject of frequent investigation by the majority of RNA biomarker projects, but also help to unravel qualitative aspects of gene expression, such as alternative splicing or alternative poly-adenylation events, that also affect protein composition and RNA stability, with direct phenotypic consequences. These aspects make complementary, rather than competitive, the distinct transcriptome interrogation approaches. The combination of large-scale transcriptome interrogation tools will facilitate the investigation of the most complex questions related to diseases of the nervous system (reviewed in Mirnics et al. 2001) and together with new and large-scale DNA sequencing approaches available today, have the potential transfigure the importance of RNA markers for psychiatric diseases.

2.1) Expressed Sequence Tags Analysis (EST):

One of the early approaches used for large-scale gene discovery and analysis was based on the partial sequencing of clones derived from cDNA libraries as proposed by the group of J. Craig Venter, in 1992 (Adams et al. 1992). Due to its capacity of generating relatively long reads over cDNA sequences, this approach is still very useful for gene discovery, alternative splicing, alternative poly-adenylation, polymorphism and mutation analysis, being extremely rich sources of transcribed biomarkers.

EST analysis starts with the extraction of the RNA of interest (mRNA or total RNA, depending on the approach used) that is going to be cloned into sequencing vectors. The

standard approach consists of using oligo-dT primers for the synthesis of the first cDNA strand, followed by the second strand synthesis and the cloning of double stranded cDNA molecules in the sequencing vectors, usually with the help of adaptors for a directional cloning. In this aspect, there is a major difference between EST and SAGE (to be described later), as ESTs usually aims to clone the entire, or the most complete cDNA fragment, while SAGE focus on very small fragments from a defined portion of the transcript. The cDNA clones are then sequenced, from one or from both ends. In non-normalized libraries, the number of times a certain gene is sequenced usually corresponds to its frequency in the original RNA pool. Also, as the fragment sequenced is usually long (500-800nt), sequence analysis provides not only a unambiguous gene identification, but also provides data useful for the observation of transcriptional diversity, such as polymorphisms, mutations or alternative splicing and alternative polyadenylation events.

The major limitations of the EST approach are related to sequencing costs required for broad transcriptome coverage. Each clone sequenced allows the study of a single transcript and due to the high level of expression of some transcripts, sequencing redundancy of the most abundant transcripts could be a limitation. Another drawback is the biased distribution of the ESTs towards the ends of the transcripts, which reduces the coverage of the whole gene. While the reduction on DNA sequencing costs is now a reality, the alternative approaches that have been described to circumvent sequencing redundancy and positional biases of ESTs inevitably abolish its use in the detection of relevant fluctuations in gene expression (Soares et al. 1994, Dias-Neto et al. 2000, Camargo et al. 2001).

In neuropsychiatry, ESTs have been used in the study of Parkinson's disease (Lu et al. 2005 (a), Kim et al. 2006) and epilepsy (Avedissian et al. 2007). ESTs have also been

used in the study of brains of schizophrenic patients and a few thousand sequences have been generated and are publicly available in the EST database at the NIH (www.ncbi.nlm.nih.gov/dbEST). Most brains used for cDNA libraries construction were obtained at the Stanley Neuropathology Consortium, and include the frontal lobe of a suicide schizophrenic, male, 34 years old (3,699 ESTs available), as well as a hippocampus (a pool of three schizophrenic patients), subtracted from a pool of three mentally normal individuals. All sequences are available in public databases, and can be used to point a number of transcriptional aspects present in these brain samples. In SCZ, ESTs were also useful for the analysis of new gene polymorphisms in the 14-3-3 eta chain gene (Bell et al. 2000).

2.2) cDNA microarrays:

The development of cDNA microarrays has provided one of the most powerful tools to the large-scale gene expression investigation in human diseases. Through the use of these arrays, global gene expression analysis could be used as valuable tool to obtain major insights into diagnosis, progression, prognosis and response to therapy for a number of human diseases. The basic technologies used for cDNA microarrays were first developed to evaluate gene expression. However, improvements of the initial methods now permits more intricate and widespread uses, including mutation analysis in expressed genes (Klevering et al. 2004, Tennis et al. 2006, Van Bogaert et al. 2007), gene sequencing and polymorphism analysis (Kozal et al. 1996, Günthard et al. 1998) and analysis of splicing events and copy number variations (Cuperlovic-Culf et al. 2006, Hughes et al. 2006, Blencowe 2006, Cowell and Hawthorn 2007). The analysis of gene expression using cDNA microarrays is one of the most used applications of the gene-chip technology as it permits the rapid,

simultaneous and sensitive analysis of a large number of biological samples for the concurrent expression of thousands of genes (Mirnics et al 2001, Magic et al. 2007). Recent developments allowed the investigation of larger fractions of the transcriptome, increasing the accuracy of cDNA microarrays, and reducing its costs, permitting microarrays to be used as a common tool in the search of markers of human diseases. These studies are often carried out in conjunction with other methods that confirm the differential expression detected with microarrays, including Northern blots and quantitative real-time PCR (QPCR).

The concepts that lead to the development of cDNA microarrays were realized soon after the first description of the double helix by Watson and Crick, in 1953. After the depiction of the DNA structure, it was realized that the two DNA strands could be separated by heat or alkali treatment, in a reversible process that underlies all the methods based on DNA hybridization. It was also observed at that time, that some degree of sequence complementarity was required during the hybridization of two sequences involved in the duplex formation. Double-strand denaturation, sequence complementarity and renaturation capability were concepts that emerged during this period and allowed the development of analytical methods based on DNA hybridization, which were quickly incorporated into a range of biological investigations. Later on, on the mid-1970s the potential of the recombinant DNA technology was realized due to a number of factors, including the capability of detecting specific clones in genomic or cDNA libraries (Grunstein and Hogness 1975, Benton and Davis 1977), using hybridization. Thus, bacterial or phage clones carrying plasmids with different inserts, could be screened and selected on membranes, exploring the concept of anchoring nucleic acids to a solid support for analysis by hybridization with radioactive-labeled probes.

Modern microarrays were developed from these key basic concepts, and now employ microscopic dots, spotted on glass slides, revealed with fluorescent probes. The most used platforms consist of a set of pre-defined arrays of DNA molecules (such as oligonucleotides, cDNA clones or PCR products) that are usually spotted onto glass slides, as well as labeled cDNAs, derived from RNAs of the samples of interest. The labeled cDNA molecules are hybridized against the elements on the arrays and the detection is usually made using fluorophores such as Cy-3 or Cy-5. Once the hybridization step is completed, the glass slides are scanned to obtain digital images of the experimental results. These digital images contain hundreds or thousands of points or "spots". Each spot represents a probe, and usually, many probes are available for each transcript. Gene expression levels are then quantified with the help of software designed for image-analysis. After processing the hybridization results of thousands of probes, representing thousands of genes, the typical goal is to find statistically significant up- or down-regulated transcripts.

The technique is sensitive and can detect gene expression fluctuations for most of the transcripts. Commercially available cDNA microarrays can detect as few as one in 250,000 mRNA copies (Mirnics et al., 2001). This level of sensitivity should allow the detection of rare mRNA species in a pool of transcripts found in a typical tissue sample (Lockhart et al. 1996, Bertucci et al. 1999, Kane et al. 2000). However, even with this high sensitivity, many low-abundance transcripts remain undetected. Unfortunately, increasing the absolute amount of the hybridized target usually will not increase the sensitivity of the microarray – as the relative abundance of the transcript within the RNA pool, coupled with microarray probe characteristics, will influence the detection limit for each individual transcript. Thus, transcripts which expression is restricted to a small subpopulation of neurons are effectively diluted by the most abundant ones and remain undetectable. Consequently, even if one had

access to the entire transcriptome on a microarray, the complexity of gene expression in the brain would preclude real global gene expression profiling.

Advantages of microarrays include its high degree of automation, the requirement of relatively small RNA amounts, as well as the capability of simultaneous analysis of thousands of genes in a single experiment, at a relatively low cost (around US$500/slide). However, as each probe is capable of evaluating only its corresponding transcript, the distinct probe properties (such as its GC content, self-complementarity, and its location in the transcript) can affect its hybridization capabilities. Thus, the expression data derived this way is relative rather than absolute, and gene-expression measurements made by microarrays still need to be confirmed by other methods. Due to the relative low cost, and the availability of a number of commercial platforms, cDNA microarrays are one of the most popular approaches to investigate gene expression.

In psychiatry research, microarray analysis have provided a wealth of information to help uncover complex biological processes, to better understand the pathogenesis of many diseases, and to discover novel potential biomarker panels, to mention a few. Transcriptomes of different brain areas have been investigated by many groups, and the results have contributed to the definition of the set of genes expressed in diseased brains, pointing to a potentially relevant set of RNA markers. Most of the markers described in this chapter have been discovered by using microarray analysis.

2.3) Serial Analysis of Gene Expression (SAGE):

Gene expression patterns in the human brain exceed the complexity of many other organ systems. The degree of difficulty in the analysis of such patterns is magnified in the investigation of psychiatric disorders, which appear to result from the interplay of

polygenic and epigenetic factors on multiple brain circuits. Thus, in many situations of the psychiatry research, it would be important to use techniques that do not require an *a priori* definition of the genes that are going to be investigated. This is one of the most interesting aspects of SAGE.

SAGE (Serial Analysis of Gene Expression) is a DNA sequencing-based technique, described by Velculescu et al, in 1995 (Velculescu et al, 1995), based on the sampling of short cDNA sequences (called tags) ideally gene-specific, from a population of cells or tissues. The presence and the sequence of these gene tags are determined by the occurrence and location of digestion sites of frequent cutter restriction enzymes. SAGE theory rests in three basic principles: First, considering a traditional SAGE experiment, a theoretical tag of 14nt – 4 nt corresponding to the restriction enzyme site, followed by the 10 nucleotides downstream - can generate 4^{10} (1,048,576) different tags, which in theory are capable to discriminate most of human transcripts (Patino et al. 2002). Second, tag concatenation allows a faster and less-expensive determination of the tag-sequence *via* cDNA sequencing, allowing the analysis of thousands of genes in a few sequencing reactions; and third, the number of times each tag is sequenced should reflect the expression level of its correspondent gene (Knox and Skuce 2005), allowing the generation of gene abundance lists, categorizing the expression of each transcript in a absolute fashion.

SAGE requires the extraction of the RNA samples to be compared, followed by the construction and sequencing of SAGE libraries containing tags concatamers. After sequencing a few thousand clones of the SAGE libraries, the frequency of the tags (which are derived from the collection of genes expressed in the studied samples) corresponds to the frequency of the respective gene in the original sample. Due to the concatenation of

small fragments for serial sequencing, tag redundancy is used to estimate transcript abundance, and does not represent a limitation as seen in the EST approach.

The construction of SAGE-libraries is based in a chain of enzymatic steps. Briefly, the process starts with the synthesis of cDNA molecules, primed by a biotinylated oligo dT coupled to magnetic beads. The cDNAs are magnetically captured and digested with a frequent (4-bp) cutter restriction enzyme, named anchoring enzyme (usually *NlaIII*). After this digestion, the digested fragments that are not coupled to the oligo-dT in the magnetic columns are washed away, and only the most 3' end fragments remain. These are subjected to the ligation of adapters containing the restriction site of other enzymes, used as tagging enzymes. A tagging enzyme is a type IIS restriction endonuclease (*BsmfI* is typically used), an enzyme that cuts the DNA fragment at a defined distance of its recognition site (up to 20 base pairs). To illustrate, the restriction enzyme *BsmfI* recognizes the sequence GGGAC and will cleave the DNA strand 10 nt downstream. As the DNA is cleaved, it is released from the bead, and the tags are ligated to each other to create ditags. These tags are then concatenated and cloned in sequencing vectors, constituting a SAGE library, ready to be used for serial DNA sequencing. The detailed protocol of SAGE as well as more information can be found at the SAGEnet website (http://www.sagenet.org/protocol/MANUAL1e.pdf - accessed in 30 Dec 2007) as well as in a recent review by Hu and Polyak (Hu and Polyak 2006).

A typical SAGE-library contains thousands of clones, each one containing a few dozens of small transcript tags (14 bp) cloned in tandem. In a single sequencing run, as many as 25-30 gene tags can be obtained from a clone, and 2,000 to 3,000 tags can be produced after sequencing a single 96 well plate. After extracting and counting the individual tags, a SAGE-tag list is produced. The tags are mapped to their genes, and their

frequency corresponds to the abundance of the correspondent gene in the original RNA pool. Thus, SAGE is an open platform that does no require an *a priori* list of genes to be investigated.

Another important aspect of SAGE regards its quantitative power. A usual SAGE experiment involves the construction and sequencing of a pair of SAGE libraries, allowing transcriptome comparison in two different situations, giving statistic significance to each compared transcript. This type of data permits a direct comparison between different experiments, laboratories, experimental systems, and data types, a crucial aspect of the database dependent analysis of biological systems that is required by functional genomics approaches. With the advent of the new DNA sequencing technologies (Margulies et al. 2005, Nielsen et al. 2006) SAGE lists with millions of tags can be generated in a few days, and data from different laboratories or experiments can be directly compared.

Maybe the most significant problem of SAGE is the generation of ambiguous tags, which could make difficult the interpretation of data. It is estimated that around 30% of the regular SAGE-tags can be mapped to multiple human transcripts, and the correct interpretation of gene regulation in the sample requires tag-extension approaches or alternative SAGE methods (reviewed in Wang 2007). One way to overcome this limitation is to generate longer (and thus, more specific gene tags) as described (Saha et al. 2002). Another limitation of SAGE is the inability of tagging some genes, due to the absence of required sites for the anchoring enzyme in a fraction of the transcripts. It has been calculated that this latter problem would halts the proper analysis of expression of 3-5% of the human genes. Due to the high costs, and many days required for library construction and sequencing SAGE libraries, this technique has been poorly used in the study of transcriptome alterations in psychiatric diseases.

The published gene expression analyses using SAGE in models to study neuropsychiatry are limited to a few papers evaluating drug-response and gene expression (Ouchi et al. 2004, Cai et al. 2005), or in models for epilepsy (Hendriksen et al. 2001, Arai et al. 2003) or Parkinson's disease (Ryu et al. 2002, Ryu et al. 2005). Few studies have been published with human samples, and are currently limited epilepsy (Ozbas-Gerçeker et al. 2006), bipolar disorder (Sun et al. 2001), Parkinson's (Noureddine et al. 2005) or Alzheimer's disease (Xu et al. 2007). After the recent development of new DNA sequencing technologies, such as the sequencing by synthesis using pyrosequencing protocols optimized for solid supports (Margulies et al. 2005) we can expect to see a rebirth of sequencing-based RNA biomarker discovery in the coming years. These technologies dramatically reduced sequencing costs, and allow the generation of more than 400,000 reads in a single sequencing run. Less expensive sequencing will make possible the sequencing of hundreds of thousands of ESTs, and millions of SAGE tags in a single day, with a strong impact in the discovery of RNA-biomarkers.

A comparative overview of the most important advantages and disadvantages of cDNA microarrays, SAGE and EST analysis is presented in Table I.

Table I – Advantages and disadvantages of three large-scale transcriptome investigation approaches.

Technique	Main Advantages	Main Disadvantages
EST analysis	- The higher informational content (quantitative/qualitative) - More precise gene identification - Excellent to provide qualitative alterations in gene expression (such as alternative splicing) - Allows the analysis of gene polymorphisms and mutations - Excellent tool for gene finding.	- cDNA library preparation is a complex step - Large amounts of RNA are required - High cost for large-scale sequencing when a broad transcriptome coverage is required - High bioinformatics requirements - Low-throughput method
cDNA microarrays	- Low cost after device installation - Large-scale evaluation of thousands of transcripts. - Relatively small RNA amounts - Low bioinformatics requirements - Method highly automated - High-throughput - The most characterized method	- Low quantification range - Allows the evaluation only of the transcripts present in the array. - Do not analyze unknown genes - cDNA may hybridize to similar probes (adjustable problem) - Require special device to array analysis - Requires complex statistics analysis.
SAGE	- Allows the analysis of unknown genes - Directly estimates gene abundance level - Expression data is absolute - Excellent quantification range - High Throughput Method - Do not require special devices - Results from any new experiment are directly comparable to other SAGE data	- SAGE library preparation is a complex step - Tags can be ambiguous - Some transcripts may lack the anchoring enzyme site - Poor detection of rare transcripts - Expensive and cumbersome DNA sequencing (applying traditional SAGE method). - High bioinformatics requirements

3. Searching for RNA markers in schizophrenia brain samples

Transcriptome studies in neuropsychiatric diseases indicated consistent alterations in the expression of many genes. In many cases, independent studies, dealing with different samples and distinct experimental platforms, have consistently found alterations in the same genes, or in the same pathways, strongly suggesting the implication of discrete physiological clusters in the pathophysiology of certain diseases. This is a strong suggestion that transcriptional alterations may be very relevant not only to propose appropriate biomarkers, but also to contribute to the comprehension of the biological basis of neuropsychiatric diseases.

In the next pages we will briefly present the regulatory clusters more consistently identified in gene expression studies in SCZ, which constitute the most promising RNA markers for this disease.

3.1) Oligodendrocyte-related genes

The main function of the oligodendrocytes is the formation of myelin sheath units around the axons of the neurons in the central nervous system. Myelin is an electrically-insulating phospholipid layer that surrounds the axons of many neurons and greatly facilitate axonal signal by increasing the speed at which the electrical impulses are propagated and by preventing the electrical current from leaving the axon. Due to the key role of myelin, oligodendrocytes have been an important focus in the study of many neurological and neuropsychiatric diseases. In the grey-matter, oligodendrocytes are more numerous than astrocytes and microglia (Dai et al. 2003), and it has been estimated that oligodendrocytes constitute about 51% of cells around the soma of large neurons in the

human cortex (Polak et al. 1982). The atrophy of pyramidal neurons in the hippocampus, which has been also reported in the pre-frontal cortex (PFC) in SCZ, can be caused by the loss of these important cells (Benes et al. 1991, Arnold et al. 1995, Zaidel et al. 1997, Rajkowska et al. 2001). Whereas the abnormal distribution and decreased density of oligodendrocytes in frontal regions of SCZ brains was observed in histological studies (Uranova et al. 2001), the search for RNA markers in different regions of SCZ brains revealed the altered regulation of many oligodendrocyte-related genes, most involved with myelin-homeostasis, suggesting the important role of myelinization and oligodendrocytes in SCZ. Alterations in the metabolism of oligodendrocytes are among the most consistent SCZ biomarkers.

One of the first publications that reported the altered regulation of oligodendrocytes in SCZ was Hakak et al (Hakak et al. 2001), using cDNA microarrays in the analysis of dorsolateral pre-frontal cortex (DPFC) pools of controls and medicated chronic SCZ patients. The findings of this report suggest that oligodendrocytes can be a specific cell type functionally deficient in SCZ. This suggestion was subsequently reinforced by independent analysis conducted by another 4 groups, also using cDNA microarrays to compare RNAs from SCZ and controls, using RNA samples extracted from DPFC or other brain regions (Tkachev et al. 2003, Prabakaran et al. 2004, Aston et al. 2004, Katsel et al. 2005, Arion et al. 2007), and by another study that evaluated the expression of selected olygodendrocyte-related genes using QPCR (Dracheva et al. 2006). Some of the oligodendrocyte-related genes found by these groups were recently confirmed by McCullumsmith et al (McCullumsmith et al. 2007) using *in situ* hybridization. Some of the most relevant papers published on RNA analysis in SCZ, as well as the brain regions and platforms used in the

study are presented in Table II. The most consistent oligodendrocyte-related gene expression alterations, identified by at least two groups, are listed in Table III.

Table II: Analysis of gene expression of oligodendrocytes-related genes of postmortem SCZ brain tissue

Reference	Type of Analysis	Subjects	Brain region
Hakak et al. 2001	Microarrays	12 SCZ; 12 controls	Pre-frontal cortex (BA46; left hemisphere; gray matter)
Tkachev et al. 2003	Microarrays	15 SCZ; 15 Bipolar; 15 controls	Pre-frontal cortex (BA9; gray/white matter)
Prabakaran et al. 2004	Microarrays	54 SCZ; 50 controls	Pre-frontal cortex (BA9 gray/white matter)
Aston et al. 2004	Microarrays	12 SCZ; 14 controls	Temporal Cortex (Middle Temporal Gyrus - BA21)
Katsel et al. 2005	Microarrays	16 SCZ; 14 controls	Superior frontal gyrus (BA8) Frontal pole (BA10) Insular cortex (BA44) Dorsolateral prefrontal córtex (BA46) Anterior cingulate (BA: 24/32) Posterior cingulate (BA: 23/31), Parietal (BA: 7) Inferior temporal gyrus (BA20) Middle temporal gyrus (BA21) Superior temporal gyrus (BA22) Parahippocampal gyrus (BA 36/28) Occipital (BA: 17) Hippocampus Caudate nucleus Caudate putamen
Dracheva et al. 2006	qPCR	I- 30 SCZ; 25 Controls II- 24 SCZ; 21 Controls III- 23 SCZ; 20 Controls IV- 24 SCZ; 19 Controls	I- Cingulate Gyrus (Brodmann area 24/32) II- Hippocampus III- Caudate Nucleus IV- Caudate Putamen
McCullumsmith et al. 2007	*in situ* hybridization	41 SCZ; 34 Controls	Anterior Cingulate Cortex
Arion et al. 2007	Microarrays and qPCR	12 SCZ; 12 Controls	Pre-frontal cortex (BA46; left hemisphere; gray matter)

Table III: Oligodendrocyte-related genes presenting altered expression in SCZ brain tissues.

Gene symbol	Gene name	Gene alteration described by:
CNP	2',3'-cyclic nucleotide 3' phosphodiesterase	Hakak et al. 2001 Tkachev et al. 2003 Prabakaran et al. 2004 Aston et al. 2004 Katsel et al. 2005 Dracheva et al. 2006 McCullumsmith et al. 2007
MAG	Myelin-associated glycoprotein	Hakak et al. 2001 Tkachev et al. 2003 Aston et al. 2004 Katsel et al. 2005 Aberg et al. 2006b Dracheva et al. 2006 McCullumsmith et al. 2007
ERBB3	v-erb-b2 erythroblastic leukemia viral oncogene	Hakak et al. 2001 Tkachev et al. 2003 Aston et al. 2004 Katsel et al. 2005
TF	Transferrin	Hakak et al. 2001 Tkachev et al. 2003 Prabakaran et al. 2004 Katsel et al. 2005 Aberg et al. 2006b McCullumsmith et al. 2007 Arion et al. 2007
GSN	Gelsolin	Hakak et al. 2001 Prabakaran et al. 2004 Katsel et al. 2005
MAL	T-lymphocyte maturation-associated protein	Hakak et al. 2001 Aston et al. 2004 Katsel et al. 2005
CLDN11	Claudin 11; Oligodendrocyte specific protein	Tkachev et al. 2003 Katsel et al. 2005 Dracheva et al. 2006
MOG	Myelin oligodendrocyte glycoprotein	Tkachev et al. 2003 Katsel et al. 2005 Arion et al. 2007
PLP	Proteolipid protein	Tkachev et al. 2003 Aston et al. 2004 Aberg et al. 2006b
PLLP/ TM4SF11	Plasmolipin or Transmembrane 4 superfamily 11	Aston et al. 2004 Katsel et al. 2005
QKI	Quaking homolog	Aberg et al. 2006a; McCullumsmith et al. 2007

Many different large-scale studies, using different samples, different brain regions and diverse microarray platforms showed significant alterations in the expression of myelinization pathways. Many of these findings were subsequently confirmed in an individual basis, by other groups, usually with sensitive approaches such as QPCR analysis. Diverse genes related to oligodendrocyte function were confirmed in an individual basis, including 2',3'-cyclic nucleotide 3' phosphodiesterase (CNP) (Peirce et al. 2006), Myelin-associated glycoprotein (MAG) (Aberg et al. 2006, Wan et al. 2005), Gelsolin (GSN) (Xi et al. 2004), Myelin oligodendrocyte glycoprotein (MOG) (Liu et al. 2005), Neuregulin (NRG1) (Tosato et al. 2005), and many others.

It is interesting to note that the myelination of the PFC occurs in the adolescence, a period when SCZ onset is more common,. It should also be noted that demyelination diseases such as metachromatic leukodystrophy, are associated with a schizophrenic-like psychoses (Hyde et al. 1992). As suggested by Hakak et al. (Hakak et al. 2001), alterations in oligodendrocyte–axon interactions may underlie cytoarchitectural changes found in SCZ. When the consistent observation of a differential regulation of myelin-related genes is taken together with clinical and physiological aspects, oligodendrocytes appear as one of the stronger players in SCZ.

3.2) Alterations in synaptic function and plasticity

One of the first large-scale transcriptome analyses of SCZ brains was made by Mirnics et al. (Mirnics et al. 2000) that used cDNA microarrays to study gene expression in PFC samples derived from 12 SCZ and 10 control subjects. PFC, the anterior region of frontal lobes, is one of the most explored brain regions in SCZ RNA studies. This neocortical region is most elaborated in primates and provides diverse and flexible

behavioral repertoires, including the differentiation of conflicting thoughts, determination of concepts of "good and bad" and perspectives in accordance of determined actions and moderating correct social behavior. Moreover, an important function operated in PFC is personality expression (Miller and Cohen 2001, Liston et al. 2006). The platform used by Mirnics et al. (Mirnics et al. 2000) contained over 7,000 unique cDNA elements, and the most relevant findings involved the reduced expression of genes involved with of pre-synaptic function (PSYN) which were subsequently verified by *in situ* hybridization. The reduced expression of *PSYN* genes, can lead to an impaired release of synaptic vesicles at nerve terminals, causing a neurotransmission imbalance frequently observed in SCZ. Two genes showed consistent regulation in individual analysis: N-ethylmaleimide sensitive factor (NSF) and synapsin II (SYN2) in all six SCZ patients included in the study. The markers revealed by this study suggest that SCZ can be the consequence of an abnormality in pre-synaptic function.

Altered state of synaptic functions and plasticity have been enrolled as primary suspects in SCZ for many years (reviewed in Owen et al. 2005). One of the major evidences of a synaptic imbalance is the hyper-dopaminergic state presented by SCZ patients, which has been proved after the demonstration that dopaminergic agonists can induce "psychotic-like status" and after the observation that the potency of an antipsychotic drug is directly proportional to its ability to block dopamine receptors (Seeman et al. 1975). Moreover, an abnormal neurodevelopment, including synapse formation, could be one of the main causes of this hyper-dopaminergic state (Raedler et al. 1998). The synaptic defects in SCZ brains may lead to deficits in episodic memory, malfunction of hippocampal circuitry, and anomalies of axonal sprouting (Ben-Shachar and Laifenfeld 2004).

Transcriptome studies revealed the differential expression of synapse-related proteins in the brains of SCZ patients and have reinforced the involvement of this pathway in SCZ. Myristoylated alanine-rich C kinase substrate, growth-associated protein-43, superior cervical ganglia-10, and neuroserpin are genes involved in neuronal development and the modulation of synaptic plasticity (Aigner et al. 1995, McNamara and Lenox 1997), which have been found to be upregulated in SCZ brains (Hakak et al. 2001).

Vawter et al. (Vawter et al. 2001), while studying middle temporal gyrus, cerebellum and PFC pools of SCZ patients, showed the alteration of rab3c, glutamate receptor ionotropic AMPA2, neuroserpin, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide, and calmodulin 3. More recently, Aston et al. (Aston et al. 2004) described the downregulation of SYN2 and synaptojanin 1, in SCZ PFC. The list of these synapsis-related genes may also include NRG1 (a protein also involved in oligodendrocyte function) which is released from glutamate terminals and regulates NMDA glutamate receptors. A synthesis of the more solid findings of synapsis-related gene alterations found in SCZ can be found in Table IV.

Table IV: Synapsis-related genes revealed as differentially expressed in transcriptome studies of SCZ brain tissue.

Reference	Gene Symbol	Gene name
Hakak et al. 2001	MARCKS	Myristoylated alanine-rich C kinase substrate
	GAP-43	Growth-associated protein-43
	SCG-10	Superior cervical ganglia-10
	SERPINI1	Neuroserpin
Mirnics et al. 2000	NSF	N-ethylmaleimide sensitive factor
	SYN2	Synapsin II
Vawter et al. 2001	RAB3C	RAB3C, member RAS oncogene family
	AMPA2	Glutamate receptor ionotropic AMPA2
	SERPINI1	Neuroserpin
	YWHAH	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide
	CALM3	Calmodulin 3
Aston et al. 2007	SYN2	Synapsin II
	SYNJ1	Synaptojanin 1

These genes code for proteins that may have an impact on the function of synapses, including glutamate synapses. The identification of these genes implies that synapses might be one of the primary abnormality sites of in SCZ, with a series of downstream consequences that might affect the neural circuitry.

3.3) Energy Metabolism

Alterations in energy metabolism have been extensively described by large-scale transcriptome and proteome analysis in SCZ (Prabakaran et al. 2004, Ben-Shachar and Laifenfeld 2004, Bubber et al. 2004, Karry et al. 2004, Glatt et al. 2005, Clark et al. 2006, Martorell et al. 2006, Mehler-Wex et al. 2006). Many aspects of the energy metabolism in brain, as well as its close connection to the neuronal plasticity and synapse (reviewed in Ben-Shachar and Laifenfeld 2004) and evidences of oxidative damage in SCZ brains

(reviewed in Yao et al. 2001) strongly suggest that SCZ has an important energetic component in its pathogenesis.

Middleton et al. (Middleton et al. 2002), studying the transcriptome of SCZ PFC cortex using microarray, showed a reduction in the expression of energy-metabolism genes involved in the mitochondrial malate shuttle, mitochondrial translocases , Ketone body metabolism and others as shown in Table V. Prabakaran et al. (Prabakaran et al. 2004) using PFC from 54 SCZ and 50 controls found transcriptional alterations of genes related to energy metabolism and to oxidative stress as shown in Table V.

Ben-Shachar et al. (Ben-Shachar et al. 2004) hypothesize that the mitochondrial dysfunction in SCZ could be caused by an imbalanced dopamine metabolism in SCZ brains. The hyper-dopaminergic state generates many dopamine oxidized metabolites, which consequently inhibits the mitochondrial respiratory system.

Table V: Energy metabolism-related genes revealed as differentially expressed in transcriptome studies of SCZ brain tissue.

Reference	Gene Symbol	Gene name
Middleton et al. 2002	GOT2	Glutamic-oxaloacetic transaminase 2
	MDH1	Malate dehydrogenase 1
	ATP5A1	ATP synthase mitochondrial F1 complex alpha
	Azin1	Antizyme inhibitor
	CRYM	Crystallin
	OAT	Ornithine aminotransferase
	TIMM17A	Translocase of inner mitochondrial membrane 17
	OXCT1	3-Oxoacid CoA transferase
	USP14	Ubiquitin-specific protease 14
	UCHL1	Ubiquitin C-terminal esterase L1
Prabakaran et al. 2004	PKM1	Muscle pyruvate kinase
	ACADS & ACADL	Peroxisomal acyl-CoA oxidase - short- and long-chain - dehydrogenases
	ACAT2	acetyl-coenzyme A acyltransferase 2
	AGPS	ATP citrate lyase, alkylglycerone phosphate synthase
	CPT1 & CPT2	carnitine palmitoyltransferases 1 and 2
	ACO	Aconitase
	ENO	Enolase
	PDH	Pyruvate dehydrogenase
	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

3.4) Other biochemical pathways

Table VI presents an overall synthesis of large-scale analysis in SCZ brains, showing the sample size and the brain areas studied, and highlighting the most relevant regulated pathways.

Table VI: Analysis of gene expression of different brain regions of postmortem SCZ brain. We did not report here the oligodendrocyte genes, which are shown in Tables II and III.

Reference	Samples	Type of Analysis	Regulated Pathways	Most important regulated genes
Mirnics et al. 2000	11 SCZ 11Controls Pre-frontal cortex (BA9)	Microarray	* Presynaptic Function * GABA neurotransmission * Glutamate neurotransmission	* N-ethylmaleimide sensitive factor * synapsin II
Hakak et al. 2001	12 SCZ 12 Controls Pre-frontal cortex (BA46)	Microarray	* Oligodendrocyte metabolism * Synaptic plasticity * Neuronal development * Neurotransmission * Signal transduction	
Vawter et al. 2001	5 medicated SCZ 3 unmedicated SCZ 3 Controls (Pre-frontal cortex, cerebellum and middle temporal gyrus)	Microarray	* Synaptic signaling * Proteolytic functions	* Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide * Sialyltransferase * Proteasome subunit, alpha type 1 * Ubiquitin carboxyl-terminal esterase L1 * Solute carrier family 10, member 1.
Middleton et al. 2002	10 SCZ 11 Controls Pre-frontal cortex (BA9)	Microarray	* Malate shuttle * Transcarboxylic acid cycle * Ornithine–polyamine metabolism * Aspartate–alanine metabolism * Ubiquitin metabolism	* Antizyme inhibitor * Crystallin * Ornithine aminotransferase * Translocase of inner mitochondrial membrane 17 * Ubiquitin-specific protease 14 * Glutamic-oxaloacetic transaminase 2, mitochondrial * 3-Oxoacid CoA * ATP synthase, mitochondrial F1 complex * Malate dehydrogenase 1, NAD (soluble) * Ubiquitin C-terminal esterase L1 (thiolesterase)
Mimmack et al. 2002	10 SCZ 10 Controls (Japan/NZ - Pre-frontal cortex)	Microarray	High-Density Lipoproteins	* Apolipoprotein L1 * Apolipoprotein L2 * Apolipoprotein L4
Vawter et al. 2002	15 SCZ 15 Controls Pre-frontal Cortex (BA9/BA46)	Microarray		* Histidine triad nucleotide-binding protein *Uubiquitin conjugating enzyme E2N * Glutamate receptor, ionotropic,
Tkachev et al. 2003	15 SCZ 15 Controls Pre-frontal cortex (BA9/BA46)	Microarray	* Oligodendrocyte metabolism	* Proteolipid protein 1 * Myelin-associated glycoprotein * Myelin basic protein * Myelin oligodendrocyte glycoprotein * Myelin protein zero * V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 * Transferrin

Prabakaran et al. 2004	54 SCZ 50 Controls Pre-frontal cortex (BA9)	Microarray	* Energy metabolism * Oxidative stress	* Metallothionein * Platelet-derived growth factor * Erythropoietin receptors * Enzyme complexes of mitochondria * Superoxide Dismutase
Aston et al. 2004	12 SCZ 14 Controls Temporal Gyrus (BA21)	Microarray	* Oligodendrocyte metabolism * Neurodevelopment * Circadian rhythms * Signaling mechanisms	* Myelin-associated glycoprotein * Plasma membrane proteolipid * V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 * Proteolipid protein 1 * TNF receptor-associated factor 4 * Histone deacetylase 3 * Neurod1 * Period homolog 1
Katsel et al. 2005	13 SCZ 13 Controls (15 different regions related in Table II)	Microarray	* Glial differentiation * Oligodendrocyte metabolism	* Myelin-associated glycoprotein * Transferrin * Quaking Protein
Dracheva et al. 2006	30 SCZ 25 Controls Cingulate Gyrus (BA24/32) 24 SCZ 21 Controls Hippocampus 23 SCZ 20 Controls Caudate Nucleus 24 SCZ 19 Controls Caudate Putamen	Q-PCR	* Oligodendrocyte and myelin-related genes	* Myelin-associated glycoprotein * Sex determining region Y-box 10 * Claudin11 * Peripheral myelin protein 22 * 2',3'-cyclic nucleotide 3' phosphodiesterase
McCullumsmith et al. 2007	41 SCZ 34 Controls Anterior Cingulate Cortex	in situ hybridization	* Oligodendrocyte and myelin-related genes	* 2',3'-cyclic nucleotide 3'-phosphodiesterase * myelin-associated glycoprotein * Transferrin * Quaking
Arion et al. 2007	14 SCZ 14 Controls Pre-Frontal Cortex (BA46)	Microarray and QPCR	* Synaptic * Oligodendrocyte * Signal transduction * Immune/chaperone	* SERPINA3 * IFITM1 * IFITM2 * IFITM3 * CHI3L1 * MT2A * CD14 * HSPB1 * HSPA1B * HSPA1A

Taken together, the results of the gene expression studies presented here suggest that there is both neuronal and glial involvement in SCZ disease process, including alterations in the transmission and propagation of the electric impulse in the axons as well as in the synapses, together with a central energy deficit in some brain areas.

4) Searching for RNA biomarkers in peripheral blood cells

Central biomarkers identified the brains of SCZ patients are fundamental to help to uncover the biological basis of the disease and to provide new targets for the development of novel therapeutic approaches. However, the difficulty to obtain fresh brain tissues, especially for RNA studies, not only complicates the analysis of a large number of patients, but also makes it difficult to have homogeneous groups with less confounding effects (such as age, diet, *causa-mortis*, gender and use of psychotropic medications). Thus, the advantages of identifying SCZ markers in peripheral tissues are obvious and have clear implications for diagnosis and for the clinical management of the patients.

The large-scale analysis of blood transcriptome in SCZ has been done by a few groups and a number of promising biomarkers have been revealed. However, the recurring identification of biomarkers has been scarce, mainly due to the distinct platforms and the heterogeneity of the clinical samples studied. Vawter et al. (Vawter et al. 2004) analyzed the transcriptome of cultured leukocytes from 5 patients with SCZ and 9 controls using an array of brain-expressed genes. These authors found statistically significant differences in the expression of 8 transcripts. The differential expression of two of these genes (neuropeptide Y receptor Y1 gene - NPY1R and guanine nucleotide-binding regulatory protein Go-alpha -GNAO1) could be confirmed by QPCR. The circulating blood

transcriptome of 30 SCZ and 17 controls was evaluated by Tsuang et al. (Tsuang et al. 2005), while searching for peripheral RNA markers for the diagnosis of SCZ. For this analysis the authors used cDNA microarrays as a platform to suggest differentially expressed genes, and QPCR for validation. The results allowed the discrimination between SCZ and controls through linear and non-linear combinations of eight putative biomarker genes as shown in Table VII with an overall accuracy of 95%–97%. The confirmation of these findings by other groups is fundamental to validate these markers.

Altered gene expression in peripheral blood cells of SCZ patients was also evaluated by Glatt et al. (Glatt et al. 2005) in one of the most austere approaches published up to now. This study used cDNA microarray analysis to investigate RNA alterations in the brain, followed by the analysis of peripheral blood cells in a second cohort of patients and controls. A total of 177 putative markers were found in brain, and 123 putative markers were seen in peripheral blood cells. Six of these RNA markers were found in both compartments as shown in Table VII. Middleton et al. (Middleton et al. 2005) analyzed by microarray and QPCR the LBP transcriptome from 33 SCZ patients, compared with controls and bipolar patients. They found many altered genes previously described in SCZ analysis as shown in Table VII. Differential expression of Alpha7-nicotinic-acetylcholine-receptor could contribute to sensory gating, leading to a marked dysfunction that can impact employability, treatment adherence, and social skills of SCZ patients. QPCR was used by Perl et al. (Perl et al. 2006) to measure the expression of this gene in peripheral blood cells of 44 SCZ patients and compared with 16 controls, and found a downregulation of this gene ($P<0.00$) in SCZ patients. Analysis of other individual genes have been performed by Mehler-Wex et al. (Mehler-Wex et al. 2006) showing the upregulation of mitochondrial complex I 75-kDa subunit in neuroleptic-naive SCZ patients and by Numata

et al. (Numata et al. 2007) that described the upregulation of PDLIM5, gene whose product regulate intracellular calcium levels and has role in neurotransmitter synaptic vesicles. PDLIM5 gene lies on chromosome 4q22, a locus previously reported to be linked with SCZ (Mowry et al 2000). A synthesis of these findings is described in Table VII.

Table VII: Analysis of RNA biomarkers found in peripheral blood cells of SCZ patients

Reference	Method	Regulated gene in Blood previously found in Brain
Vawter et al. 2004	Microarray	* Neuropeptide Y (NPY) (Found regulated in brain by Hakak et al., 2001) * Malate Dehydrogenase (MDH1) (Found regulated in brain by Hakak et al., 2001; Middleton et al., 2002)
Tsuang et al. 2005	Microarray and QPCR	* Catalytic polypeptide-like apolipoprotein B mRNA editing enzyme 3B (APOBEC3B) * Adenylosuccinate synthetase (Adssl1) * ataxia telangiectasia mutated (ATM) * Charcot-Leyden crystal protein (CLC) * C-terminal binding protein 1 * Death-associated transcription factor 1 (Datf1) * Chemokine C-X-C motif ligand 1 (CXCR1) * S100 calcium binding protein A9 (S100A9)
Glatt et al. 2005	Microarray	* B cell translocation gene 1, antiproliferative (BTG1) (upregulated in DLPFC and downregulated in blood) * Glycogen synthase kinase 3 α (GSK3a) (downregulated in DLPFC and upregulated in blood) * Heterogeneous nuclear ribonucleoprotein A3 (HNRPA3) (upregulated in DLPFC and downregulated in blood) * MHC class II, DR β 1 (HLA-DRB1) (downregulated in DLPFC and blood) * Selenium-binding protein 1 (SELENBP1) (upregulated in DLPFC and blood)

		* Splicing factor, arginine/serine-rich 1 (splicing factor 2, alternate splicing factor) (SFRS1) (upregulated in DLPFC and downregulated in blood)
Middleton et al. 2005	Microarray and QPCR	* Alpha catenin (CTNNA1) * Neuregulin 1 (NRG1) * MAX-like protein X (TCFL4) * v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian) (ERBB2) * Sensory motor neuron derived factor (SMDF) * Cytochrome P450 family 1, subfamily B, polypeptide 1 (CYP27B1) * Quaking homolog (QKI) * Dual specificity phosphatase 6 (DUSP6).
Bowden et al. 2006	Microarray and QPCR	* Endothelial differentiation gene 2 (Edg-2) * Ezrin-radixin-moesin phosphoprotein 50 (EBP50) * Myc-associated zinc finger protein (MAZ) * Tumor Necrosis Factor Receptor 2 (TNFR2)
Perl et al. 2006	QPCR	* Alpha7-nicotinic-acetylcholine-receptor (CHRNA7)
Mehler-Wex et al. 2006	QPCR	* Mitochondrial complex I 75-kDa subunit (NDUFS1)
Numata et al. 2007	QPCR	* PDZ and LIM domain 5 (PDLIM5)

5) New classes of RNA markers in psychiatry

5.1) Alternative Splicing Studies

The vast majority of the transcriptome analyses performed during the search for RNA markers in SCZ was based on the old central dogma of "one gene, one mRNA, one protein", which clearly oversimplifies and underestimates transcriptome complexity. These studies are usually focused on quantitative alterations of gene expression, and don't consider qualitative variations that may occur. While transcriptional regulation plays important roles within a cell, post-transcriptional regulation, such as alternative splicing (AS), dramatically increases transcriptional diversity and may have remarkable consequences over proteome variety. AS plays a critical role in gene expression regulation and human diseases (Kan et al. 2001, Cartegni et al. 2002). Splicing is a cellular mechanism that joins the exons of a precursor immature RNA, removing its intronic sequences. This mechanism occurs in the spliceosome, a complex cellular compartment composed small nuclear RNAs and hundreds of proteins. Since the alternative combination of different gene exons can generate diverse protein isoforms, which can trigger different mechanisms inside the cell, the study of AS as a qualitative gene expression data has become one of the central issues in biomedical sciences (Buratti et al. 2006) including SCZ research.

Studies of AS isoforms as possible RNA markers in SCZ have been focused over a few genes, usually those traditionally studied in SCZ. An example is the Dopamine receptor D2 which produces, by AS, two receptor isoforms called D2L (for long isoform) and D2S (for short) (Giros et al. 1989). These two isoforms have distinct functions in vivo: D2L acting mainly at postsynaptic sites and D2S serves as a presynaptic autoreceptor

(Usiello et al. 2000). These subunits differentially contribute to the therapeutic actions and side effects of antipsychotic agents (Xu et al. 2002). Splicing variants are also responsible for molecular dissimilarity of genes related to a number of important SCZ pathways discussed in this chapter, including genes involved in myelinization and synapses. Good examples are the synapsins, synaptic vesicle-associated phosphoproteins that have been implicated in the control of neurotransmitter release and synaptogenesis. It should be noted that a member of this family (synapsin III) is located in human chromosome 22q, in a SCZ-susceptibility locus, and different AS isoforms have been described in the brain (Kao et al. 1998).

Quaking homolog (QKI) is development-related protein encoded by a gene highly conserved among different species. A deletion of a portion of this gene causes body tremor and severe dysmyelination of the central nervous system, with dysfunction of oligodendrocytes and a reduced expression of myelin components (Sidman et al. 1964, Ebersole et al. 1996, Hardy et al. 1996). Aberg et al. (Aberg et al. 2006(a)) showed that one of the four AS isoforms of QKI (named *QKI-7kb*) was reduced in the frontal cortex of SCZ patients. This same group showed later (Aberg et al. 2006(b)) that the reduced expression of this isoform could be correlated to the reduced expression of three tightly regulated myelin-related genes (*PLP1*, *MAG*, and *TF*) that also had a reduced expression in SCZ brain samples as compared to controls. These results indicate that QKI may be a master regulator of oligodendrocyte differentiation/maturation in the human brain and also suggest that decreased activities of myelin-related genes in SCZ might be caused by a disturbed *QKI* splicing.

Other examples of AS in SCZ include variations in the relative abundance of alternatively spliced isoforms of the gamma2 subunit of the GABA-A receptor (Huntsman

et al. 1998, Zhao et al. 2006), NCAM1 (Neural cell adhesion molecule) (Vawter et al. 2000), NMDA receptor (Clinton et al. 2003), glutamate receptor 3 (mGluR3) (Sartorius et al. 2006), and ErbB4 (a Neuregulin 1 receptor) (Silberberg et al. 2006, Law et al. 2007). These examples clearly demonstrate that this class of transcriptional variation also can be relevant in the search of RNA markers for SCZ. In the near future, the analysis of AS RNA markers in SCZ should be reinforced by the use of cDNA microarrays specifically designed to investigate these events in human transcripts, as well as by new sequencing-based approaches that may reveal this kind of event.

5.2) microRNAs

A promising class of markers that still needs to be more explored in neuropsychiatry consists of this group of small non-coding regulatory RNA molecules found from plants to viruses and animals (reviewed in Bartel 2004). The advent of tiling genomic arrays showed that a significant fraction of the non-coding genome sequence is transcribed (Kapranov et al. 2002, Cheng et al. 2005). Among these non-coding RNA molecules we find the microRNAs (miRNAs) that are considered today to be the most important transcriptional regulators of the human genome (reviewed in Zhang and Farwell 2007). Some papers suggest that miRNA are more consistent regulators than mRNA and that the study of few hundreds of these molecules can derive stronger markers than tens of thousands mRNAs used in cDNA microarray platforms (Lu et al. 2005 (b)).

miRNAs control distinct processes that lead to regulation of protein abundance, such as transcription, mRNA degradation, stability and translation. An increasing number of studies now reveal the important role of miRNAs in biological processes such as the differentiation and specificity of neurons (Vo et al. 2005, Krichevsky et al. 2006), synapse

plasticity (Schratt et al. 2006) development and maintenance of the central nervous system (Krichevsky et al. 2003, Miska et al. 2004, Giraldez et al. 2005, Lukiw and Pogue 2007, Makeyev et al. 2007). The importance of miRNAs in the regulation of these processes, as well as its abundance in the brain, makes these molecules as attractive RNA biomarkers to be explored in SCZ.

The analysis of miRNA in SCZ is still in its early days. A recent study evaluated for the first time the differential expression of miRNAs in SCZ (Perkins et al. 2007). In this study, with a custom miRNA array, the expression of 264 distinct miRNAs was contrasted in postmortem PFC tissue samples of individuals with SCZ (n = 13) or schizoaffective disorder (n = 2) and non-psychiatric patients (n = 21). The authors found 16 miRNA to be differentially expressed in PFC of patient subjects, being 94% down-regulated, what suggests an overall up-regulation of their targets. The authors concluded that genes that were commonly targeted by the regulated miRNAs were significantly clustered in 12 pathways. The most significantly pathways identified contain proteins involved in synaptic plasticity at the level of dendritic spines.

Today we know that most of the eukaryotic genome is transcribed as noncoding RNAs (ncRNAs), which play important roles in chromatin organization, gene expression, post-transcription regulation, with consequences over normal physiology and disease etiology. The increasing diversity and high expression of ncRNAs, including miRNAs, identified in the eukaryotic genome suggests a critical link between the regulatory potential of ncRNAs and the complexity of genome expression regulation.

Summary and Perspectives:

The continuous identification of alterations in genes related to synapsis, myelinization and energy metabolism strongly suggests the involvement of these elements in the pathogenesis of SCZ. However, larger studies involving distinct populations and more samples are still required to fully demonstrate the importance of markers already revealed, and to point new markers valuable for more specific endophenotypes.

The future of neuropsychiatric diseases is likely to be profoundly dependent upon the use of biomarkers that shall guide the clinicians at many steps of disease management including early diagnosis, response to therapy and disease outcome. Biomarkers that detect diseases, and predict their outcome or influence treatment choice will have tremendous importance in the future of neuropsychiatric diseases. However, to understand the significance of each biomarker and its importance in pathogenesis, many samples, comprehending diverse ethnical backgrounds, endophenotypes and disease states need to be investigated. Massive amounts of biological information will need to be investigated under a multidisciplinary approach involving clinicians, biologists, statisticians and bioinformatics in a continuous interface of genomics, transcriptomics and proteomics.

It is clear that, together with DNA and protein, RNA biomarkers will be part an optimistic scenario where more detailed and personalized data will be evaluated together, guiding researchers and clinicians to understand and to treat complex diseases.

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Capítulo 3:

Padronização para Preparação de Amostras Protéicas para Análise Proteômica de Tecidos Cerebrais Humanos

O capitulo a seguir é um artigo publicado em 2007 intitulado "The use of ASB-14 in combination with CHAPS is the best for solubilization of human brain proteins for two-dimensional gel electrophoresis" no periódico "Briefings in Functional Genomics and Proteomics" (Vol. 6; p.70-75) que descreve a padronização para a ideal determinação do proteoma de amostras de cérebros humanos, utilizada posteriormente em nossas analises proteômicas de cérebros de pacientes com esquizofrenia.

BRIEFINGS IN FUNCTIONAL GENOMICS AND PROTEOMICS. VOL 6 NO 1 70–75 doi:10.1093/bfgp/elm009

Short Communication

The use of ASB-14 in combination with CHAPS is the best for solubilization of human brain proteins for two-dimensional gel electrophoresis

Daniel Martins, Bruno Menezes de Oliveira, Alessandro dos Santos Farias, Ricardo Shiniti Oka Horiuchi, Cleyton Crepaldi Domingues, Eneida de Paula, Sérgio Marangoni, Wagner Farid Gattaz, Emmanuel Dias-Neto and José Camillo Novello

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Abstract

Protein extraction is the most important step to reveal a proteome by Two-Dimensional Gel Electrophoresis. Usually, the urea/thiourea based standard protein extraction buffer (SB) is combined with detergents with the aim of achieving better resolution and solubilization of different classes of proteins. In order to produce better gels and achieve the greatest spot resolution of Human Brain Proteins, comparisons using 2-DE of extracted proteins from Human Brain Frontal Cortex with SB constituents (7M Urea, 2M Thiourea and 100mM DTT) were made, using different detergent compositions in the buffer. SB preparations in combination with CHAPS and ASB-14 as well as with ASB-16 (reported for the first time in 2-DE experiments) have been tested. Our results confirm that the most efficient solubilizing solution for 2-DE analysis of cytosolic and membrane Human Brain Proteins is SB combined with 4% CHAPS and 2% ASB-14.

Keywords: *ASB; CHAPS; two-dimensional gel electrophoresis; protein extraction; human brain; brain*

Corresponding author. Daniel Martins, Departamento de Bioquímica, Instituto de Biologia, UNICAMP, Campinas, SP, Brazil – 13083-970, Tel/Fax: 55 – 19 – 35216654. E-mail: daniel@proteome.ibi.unicamp.br

Daniel Martins is affiliated with Laboratório de Proteômica, Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), and with Laboratório de Neurociências LIM-27, Instituto de Psiquiatria, Faculdade de Medicina da Universidade de São Paulo (USP).

Bruno Menezes de Oliveira is affiliated with Laboratório de Proteômica, Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP).

Alessandro dos Santos Farias is affiliated with Laboratório de Neuroimunologia, Departamento de Microbiologia e Imunologia, Universidade Estadual de Campinas (UNICAMP).

Ricardo Shiniti Oka Horiuchi is affiliated with Laboratório de Proteômica, Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP).

Cleyton Crepaldi Domingues is affiliated with Laboratório de Biomembranas, Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP).

Eneida de Paula is affiliated with Laboratório de Biomembranas, Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP).

Sérgio Marangoni is affiliated with Laboratório de Proteômica, Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP).

Wagner Farid Gattaz is affiliated with Laboratório de Neurociências LIM-27, Instituto de Psiquiatria, Faculdade de Medicina da Universidade de São Paulo (USP).

Emmanuel Dias-Neto is affiliated with Laboratório de Neurociências LIM-27, Instituto de Psiquiatria, Faculdade de Medicina da Universidade de São Paulo (USP) and with The University of Texas, MD Anderson Cancer Center, 1515 Holcombe Blvd, Houston, Texas 77030, USA.

José Camillo Novello is affiliated with Laboratório de Proteômica, Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP).

INTRODUCTION

Despite all the described and known limitations of two-dimensional gel electrophoresis (2-DE) regarding reproducibility and difficulty in efficient separation of low abundance proteins, low and high molecular weight proteins, highly acidic, basic and hydrophobic proteins [1, 2], this technique still remains, in combination with Mass Spectrometry (MS), one of the most important methods for revealing complex protein profiles [3]. Protocol changes have been developed that aim to overcome these limitations, however, some restrictions related to protein extraction and solubility are still technical challenges.

To reach a satisfactory 2-DE resolution and a good representation of the proteome, a high-quality protein solubilization is required, consequently protein extraction has become the most important step in proteome studies. Each particular extracted sample has peculiarities related to the different classes of proteins presented; therefore, appropriate changes in the protocol depend on the nature of the sample [4]. This work was initiated to develop a good proteome representation of human brain proteins by 2-DE.

In the extraction of proteins for 2-DE, the Urea/Thiourea-based buffer combined with a reducing agent (normally DTT or TBP) and a zwitterionic or non-charged detergent [5–8] is the most frequently used buffer. The main differences between several protocols for protein extraction are related to the nature and concentration of the detergents used.

Initially, detergents employed to solubilize membrane proteins in aqueous solution, such as Triton X-100 and Nonidet P-40, were used in protein extraction for 2-DE mainly for the solubilization of the more hydrophobic proteins. Recently, the sulfobetain CHAPS has become the most commonly used detergent [9, 10], while a new class of more polar detergents, the zwitterionic sulfobetaines with alkyl tails longer than 12 carbons, has been synthesized, specifically for protein extraction and 2-DE analysis. These detergents are known as ASB (amidosulfobetain), with type 14, with its 14 carbon atoms tail, reported in the solubilization of bovine neutrophile membrane proteins, Arabidopsis plasma membrane and *Escherichia coli* outer membrane proteins. Results have shown that ASB-14 is more efficient in urea/thiourea mixtures for solubilizing a greater number of protein classes than other detergents [11].

Extraction of brain proteins with ASB-14 has already been tested by Carboni and colleagues, in mice [12]. The present work was designed to test not only the use of ASB-14 in human brain samples but also the use of ASB-16, an analog that has never been tested in brain proteins extraction before.

MATERIAL AND METHODS

Sample preparation

Six aliquots of 50 mg of Human Frontal Cortex were ground using a Polytron device on 240 µl of six different extraction buffers, as described in Table 1. Adapting protocols from previous work, in which CHAPS and ASB detergents were used separately, herein they were also combined. An aliquot of each of the resulting protein extracts was used to determine the protein concentration, using a commercial protein assay kit (Bio-Rad, Hercules, CA, USA).

Two-dimensional gel electrophoresis (2-DE)

600 µg of each different protein extract of human frontal cortex was added to a final volume (350 µl) of a solution containing 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (v/v) carrier ampholytes pH 3–10, 70 mM DTT and 0.001% (w/v) bromophenol blue (BPB). Except for CHAPS, which was obtained from Sigma (St Louis, MO, USA), all reagents were purchased from GE Biosciences (Uppsala, Sweden). Samples were applied to IPG gel strips with a separation range of pH 3–10 (GE Biosciences). After 12 h of rehydration, IEF was carried out at 20° C, initially for 1 h at 500 V, for an additional hour at 1000 V and then for 10 h at 8000 V in an IPGphor apparatus (GE Biosciences), maintaining a limiting current of 50 µA per strip. First dimension strips were subjected to the standard reduction (10 min) and alkylation (10 min) steps prior to second dimension electrophoresis. The second dimension (SDS-PAGE) was performed on 12.5%T polyacrylamide gels, run on an SE-600 system connected to a Multitemp II refrigerating system (GE Biosciences). The run was carried out for 1 h at 60 V and subsequent hours at a constant current of 30 mA per gel until the dye front reached the bottom of the gel and the proteins were detected by a colloidal coomassie blue staining.

Table 1: Extraction buffers used in this work: composition, number of spot revealed in triplicates of 2-DE profile, average number of spots and standard deviation. Detergent concentrations used were chosen according to tests of protein solubilization, carried out in our work group (data not shown)

Detergent	Composition	Number of spots detected in 2-DE profiles triplicates	Average spots number	Standard Deviation
CHAPS	7 M urea, 2M thiourea, DTT 100mM, 1% Sigma Anti-protease Cocktail and 4% CHAPS.	966 985 977	976	9,54
CHAPS + ASB 14	7 M urea, 2M thiourea, DTT 100mM, 1% Sigma Anti-protease Cocktail, 4% CHAPS and 2% ASB14.	1207 1181 1188	1192	13,45
CHAPS + ASB 16	7 M urea, 2M thiourea, DTT 100mM, 1% Sigma Anti-protease Cocktail, 4% CHAPS and 2% ASB16.	1075 1096 1090	1087	10,82
CHAPS + ASB 14 + ASB 16	7 M urea, 2 M thiourea, DTT 100mM, 1% Sigma Anti-protease Cocktail, 4% CHAPS, 2% ASB14 and 2% ASB16.	983 972 964	973	9,54
ASB 14	7 M urea, 2M thiourea, DTT 100mM, 1% Sigma Anti-protease Cocktail and 2% ASB 14	955 937 940	944	9,64
ASB 16	7 M urea, 2M thiourea, DTT 100mM, 1% Sigma Anti-protease Cocktail and 2% ASB 16.	798 814 818	810	10,58

All gels were made in triplicate and the numbers of spots of each triplicate are shown in Table 1.

2-DE image analysis

After staining, gels were scanned by transmittance at 300 dpi of resolution using a specialized Sharp Scanner JX. Images were saved as 8 bit TIFF files. The software ImageMaster 2D version 3.01 (GE Biosciences) was used for spot detection, counting and quantification.

Statistical analysis

The statistical significance of the results was determined using a parametric analysis of variance (ANOVA corrected by Bonferoni) and *t*-test. A *P*-value <0.05 was considered to be significant.

RESULTS AND DISCUSSION

The resultant data is shown in Figure 1. Gels were made in triplicate and the number of spots showed in Figure 1 for each gel is the average of spots number from triplicates. It is possible to see that the combination of CHAPS and ASB-14 (Figure 1B) can reveal significantly more spots in the 2-DE profile, as shown by the increased number of spots with good resolution ($P = 0,0087$ versus CHAPS + ASB-16 – the most similar 2-DE profile of CHAPS + ASB-14). Comparing the number of spots of all gels of the triplicate, there is a statistically significant difference between them, proven by parametric analysis of variance (ANOVA) and *t*-test (Figure 2). Gels were divided into quadrants to better visualize the favoured areas, according to the detergent. Comparing all the samples, it is possible to visualize major differences on the basic and high molecular weight proteins quadrant (regions indicated by arrows in Figure 1).

CHAPS is an efficient detergent for soluble proteins in the presence of high amounts of urea and thiourea (7M and 2M, respectively) [13], a necessary condition in IEF experiments, since

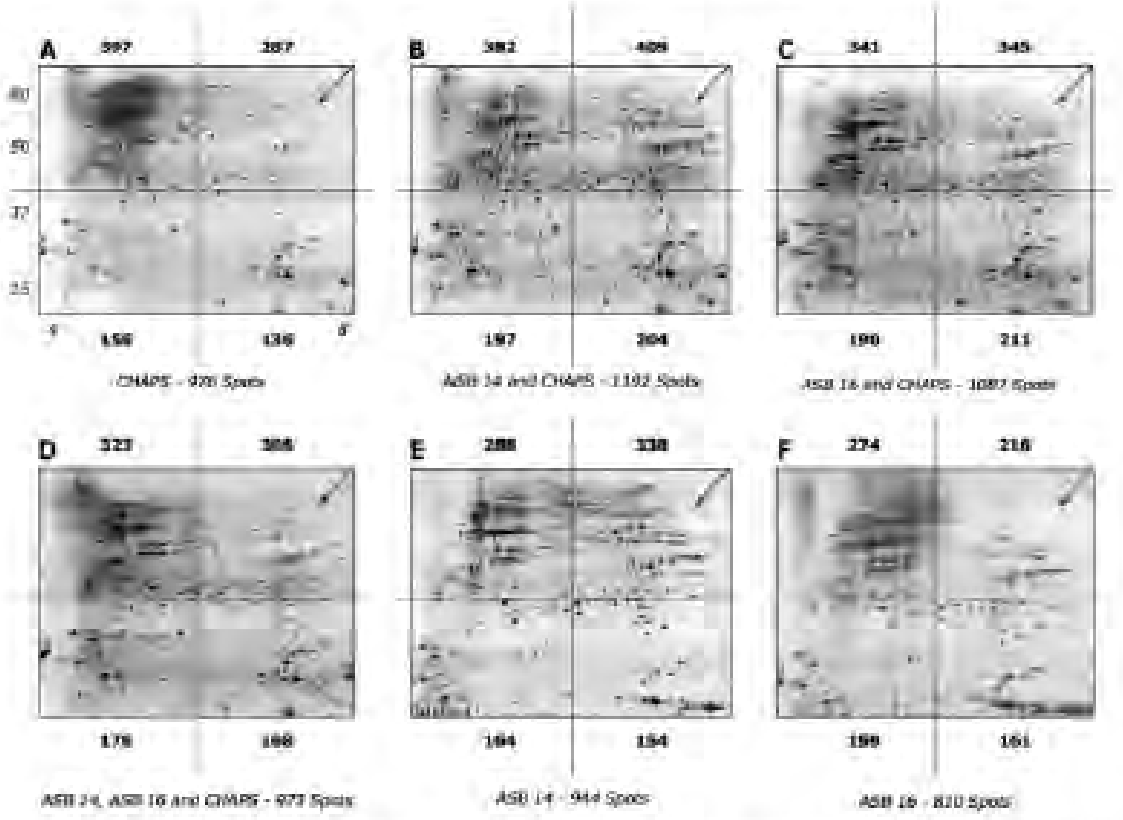

Figure 1: 2-DE profiles using different detergent composition to extract human brain proteins: (A) CHAPS; (B) Combination of CHAPS and ASB14; (C) Combination of CHAPS and ASB 16; (D) Combination of CHAPS, ASB 14 and ASB 16; (E) ASB 14; (F) ASB 16. The shown spots number means the average of spots number from triplicates. It is possible to see that the best extraction is achieved combining CHAPS and ASB- 14 in sample buffer urea/thiourea based since there are more spots, the basic proteins of high molecular weight are favoured and the spot complexity on the acid region is better resolved, compared with the other extractions. Spots were counted by ImageMaster 3.01 (GE Biosciences).

strong protein denaturants, such as SDS, cannot be used in such experiments. However, CHAPS efficiency in solubilizing hydrophobic proteins is lower in comparison to long acyl tail detergents, which are usually not compatible with high amounts of chaotropics. The solution for this problem was found by Chevallet and colleagues, who have synthesized the ASB detergents with more polar but neutral heads and longer alkyl tails (14 or more carbons). The long ASB carbon tail provides these detergents a better interaction with hydrophobic proteins, efficiently coating them. These features make it possible to achieve the maximum efficiency of the detergent, even in strong chaotropic mixtures [11].

As shown by our results, the combination of CHAPS' advantages over soluble protein solubilization with ASB-14 over hydrophobic protein solubilization may provide a good solution for proteome 2-DE profiles of human brain proteins. The data obtained by Stanley and colleagues in human myocardium [14] support our findings, since with the use of ASB-14 more basic proteins are revealed, such as triosephosphate isomerase (pI 6.4) and Mn-superoxide dismutase (pI 8.3), when compared with the use of other detergents.

It might be predicted that using ASB-16, whose carbon tail is longer than that of ASB-14, would produce a better resolution of basic proteins than ASB-14 but experimentally and statistically, we saw no evidence of this, even when ASB-16 was used in combination with CHAPS (Figure 1C). To better explain this fact, Chevallet and colleagues asserted

that subtle chemical modifications indice changes in the major physical–chemical properties of these detergents as well as in their solubilizing properties, as is often observed in detergent experiments.

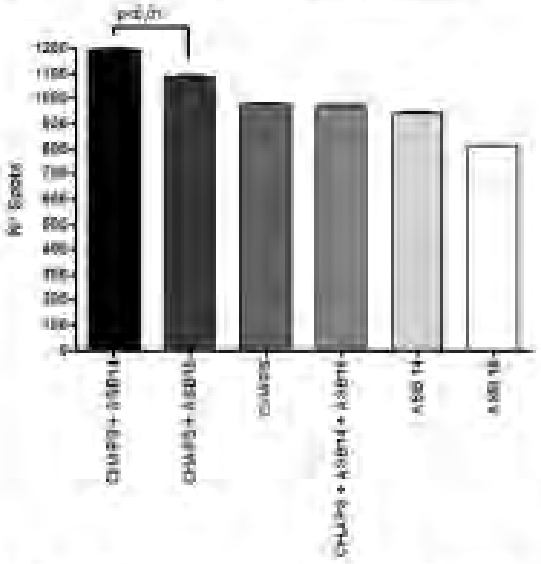

Figure 2: CHAPS + ASB-14 and CHAPS + ASB-16 were the treatments that have shown better resolution combined with higher number of detectable spots (1192 and 1087, respectively). Both were high significant compared with all other treatments (at least $P < 0.001$). Nevertheless, CHAPS + ASB-14 was even better then CHAPS + ASB-16, showing significant difference ($P < 0.01$) on the number of detectable spots.

To serve as a control, we have extracted proteins without CHAPS (Figure 1E and 1F) and not so many proteins were resolved when compared to the runs in the presence of solubilization buffer with CHAPS. This can be explained due to the known efficiency of CHAPS in solubilizing proteins in urea/thiourea mixture, as discussed earlier.

Our data also show that the solubilization of acidic and high molecular weight proteins is better achieved when the combination of ASB-14 and CHAPS was adopted, making this extraction particularly well suited to the extraction of human brain proteins.

To better visualize the differences in the basic protein regions, we have carried out a basic 2-DE profile in triplicate, from pH 6–10, for comparison with the best extraction obtained for pH 3–10 profile (combination of ASB-14 and CHAPS) and with the standard buffer (only CHAPS) profile. The results in Figure 3 confirm our findings, since there are significantly more spots in the combination profile of ASB-14 with CHAPS ($P < 0.001$) and the gel is highly resolved for spot visualization mainly in the left area of high molecular height (squared pointed region).

CONCLUSION

Our data asserts that a better resolution in a 2-DE profile of human brain proteins can be achieved with the combined use of 4% CHAPS and 2% ASB-14 in

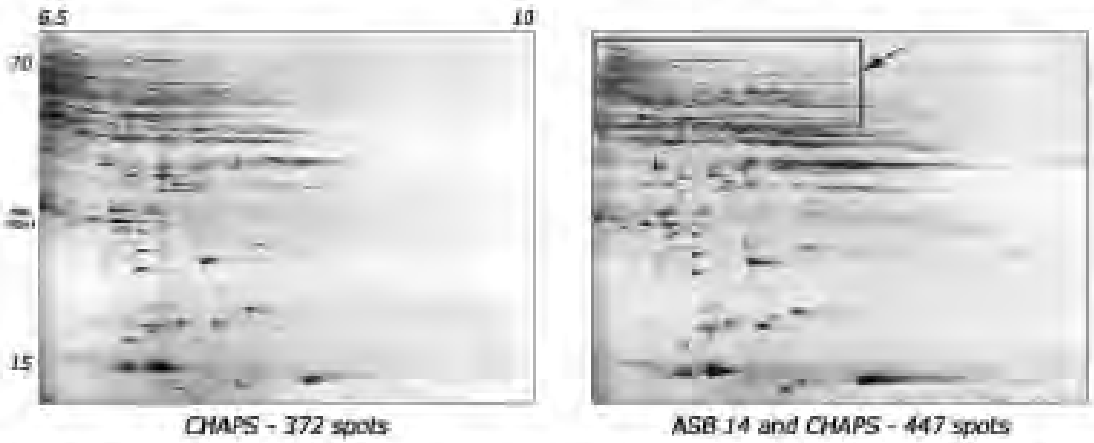

Figure 3: Comparison between 6,5–10 profile of the standard buffer extraction (CHAPS) and buffer extraction with CHAPS and ASB-14. The shown spots number means the average number of gels triplicates and the spot number of triplicates were 381, 364, 369 to CHAPS gels and 428, 456, 477 to CHAPS + ASB-14 gels. The difference in spot number is high significant ($P < 0.001$). It is possible to see, as in the 3–10 profiles, that the combination of CHAPS and ASB-14 provides a better representation of the proteome, since they reveal more and better resolved spots.

the extraction buffer, what will lead to a better qualitative proteome representation (more spots) and enhanced quantitative features, since the proteins on the acidic region are better revealed due to the reduction of spots complexity.

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Capítulo 4:

Análise Comparativa do Proteoma de Córtex Pré-Frontal Cerebral de Pacientes com Esquizofrenia e Controles Utilizando Eletroforese e Espectrometria de Massas.

Através de um manuscrito submetido ao periódico "Schizophrenia Research", descrevemos os dados por nós revelados do proteoma comparativo de córtex pré-frontal de pacientes com esquizofrenia e controles por Eletroforese de Duas Dimensões em Gel de Poliacrilamida e Espectrometria de Massas.

Proteomic analysis of pre-frontal cortex indicates the involvement of cytoskeleton, oligodendrocyte, energy metabolism and new potential markers in schizophrenia

Daniel Martins de Souza[1,2,3], Wagner F. Gattaz[1], Andrea Schmitt[4,5], Giuseppina Maccarrone[3], Eva Hunyadi-Gulyás[3], Marcos N. Eberlin[6], Gustavo H. Souza[6], Sérgio Marangoni[2], José Camillo Novello[2], Christoph W. Turck[3], Emmanuel Dias-Neto[1,7].

1- Laboratório de Neurociências – Instituto de Psiquiatria – Faculdade de Medicina da USP – Rua Dr. Ovídio Pires de Campos, no 785, São Paulo, SP, CEP 05403-010, Brazil.
2- Laboratório de Proteômica – Departamento de Bioquímica - Instituto de Biologia – UNICAMP – CEP 13083-970 - Campinas – SP - Brazil
3- Max Planck Institute of Psychiatry – Munich – Germany.
4- Department of Psychiatry, Von Siebold Str. 5, University of Goettingen, 37075 Goettingen, Germany.
5- Central Institute of Mental Health, J5, 68159 Mannheim, Germany.
6- Thomson Mass Spectrometry Laboratory, Institute of Chemistry, State University of Campinas, UNICAMP, Campinas, SP 13083-970, Brazil.
7- Present address: University of Texas, MD Anderson Cancer Center, 1515 Holcombe Blvd, 77030, Houston, Texas, USA.

Corresponding Author:
Emmanuel Dias-Neto
Laboratório de Neurociências
Instituto de Psiquiatria - Faculdade de Medicina - Universidade de São Paulo
R. Dr. Ovídio de Campos, s/n - Consolação
05403-010, São Paulo, SP - Brazil
Phone 55-11-3069.7267, 3069.7283
emmanuel@usp.br
edneto@mdanderson.org

Abstract:

Schizophrenia is likely to be a consequence of serial alterations in a number of genes that, together with environmental factors, will lead to the establishment of the illness. The dorsolateral prefrontal cortex (Brodmann´s Area 46) is implicated in schizophrenia and executes high functions such as working memory, differentiation of conflicting thoughts, determination of right and wrong concepts, correct social behavior and personality expression. We performed a comparative proteome analysis using two-dimensional gel electrophoresis of pools from 9 schizophrenia and 7 healthy control patients' dorsolateral pre-frontal cortex aiming to identify, by mass spectrometry, alterations in protein expression that could be related to the disease. In schizophrenia-derived samples, our analysis revealed 11 downregulated and 13 upregulated proteins. These included alterations previously implicated in schizophrenia, such as oligodendrocyte-related proteins (myelin basic protein and transferrin), as well as malate dehydrogenase, aconitase, ATP synthase subunits and cytoskeleton-related proteins. Also, six new putative disease markers were identified, including energy metabolism, cytoskeleton and cell signaling proteins. Our data not only reinforces the involvement of proteins previously implicated in schizophrenia, but also suggests new markers, providing further information to foster the comprehension of this important disease.

Keywords: schizophrenia, proteomics, mitochondria, cytoskeleton, myelinization, dorsolateral prefrontal cortex.

1) Introduction

Schizophrenia (SCZ) is considered to be multifactorial, including neurodevelopmental aspects, multiple associated genetic loci, and important environmental factors (Freedman, 2003). Besides some macroscopic brain alterations observed in brain scans of SCZ patients, the disease diagnosis is essentially clinical, with no solid definition of its molecular basis and a lack of reliable and consistent biochemical markers (DSM-IV Diagnostic and Statistical Manual of Mental Disorders – Frances et al, 1991).

Pre-frontal cortex (PFC) is an anterior region of frontal lobes located above motor and premotor areas. This neocortical region is most elaborated in primates, where it provides a diverse and flexible behavioral repertoire. Divided into dorsolateral, orbitofrontal and medial areas, PFC functions are neurologically called "executive functions" (Miller and Cohen, 2001). These functions include differentiation of conflicting thoughts, determination of concepts of good and bad, perspectives in accordance with determined actions, moderating correct social behavior, future consequences of current activities as well as working memory. An important function influenced by PFC is personality expression (Miller and Cohen, 2001). Basically, the activities of this region involve the organization of thoughts and actions according to internal aims (cognitive control) (Miller and Cohen, 2001; Liston et al, 2006). Dysfunction of the dorsolateral PFC (DLPFC) has been implicated in the pathophysiology of SCZ by many groups (Weinberger, 1996; Barch, 2005; Barbas and Zikopoulos, 2007)

In this work, we performed comparative proteomic analysis of DLPFC (Brodmann area 46) tissue of a pool of nine SCZ patients, which was compared to a pool of seven paired controls, using two-dimensional gel electrophoresis (2-DE) followed by MALDI-

TOF/TOF mass spectrometry. The putatively regulated proteins include previously identified SCZ markers, which suggest their importance in SCZ, as well as new markers, that are potentially involved in the pathobiology of SCZ.

2) Materials and Methods

2.1) Materials

All solid chemicals and solvents were from GE Healthcare (Piscataway, NJ) and of the highest purity available.

2.2) Human Dorsolateral Prefrontal Cortex samples (DLPFC)

Post-mortem brain samples from the DLPFC tissue (BA46) were collected from 9 schizophrenia patients and 7 controls free from psychiatry disorders. Brain samples were dissected by an experienced neuropathologist and deep-frozen immediately after collection. On average, the samples were collected 24.3 h after death.

All samples were obtained from Central Institute of Mental Health (Mannheim, Germany). All cases and controls were whites and born in Germany. All SCZ patients had been long-term inpatients at the Mental State Hospital Wiesloch, Germany, and have been diagnosed *ante mortem* by an experienced psychiatrist according to the criteria of DSM IV of the American Psychiatry Association for Schizophrenia (American PA, 1994). For each patient the antipsychotic treatment history was assessed by examining the medical charts and calculated in chlorpromazine equivalents (CPE). All patients and controls underwent neuropathologic characterization to rule out associated neurovascular or neurodegenerative disorders such as Alzheimer's disease and multi-infarct dementia. The classification

according to Braak was stage II or less for all subjects (Braak and Braak 1991, Braak et al. 2006). Patients and controls had no history of alcohol or drug abuse, nor severe physical illness. Normal comparison subjects also had no history of psychiatric disorders. All assessment and *post mortem* evaluations and procedures were previously approved by the Ethics Committee of the Faculty of Medicine of Heidelberg University, Germany. Detailed patient information is given in Table 1.

2.3) Sample Preparation

Fifty milligrams of human DLPFC (gray matter) were individually homogenized in 1.5ml tubes with glass spheres in 200µl of 6M Guanidine·HCl and 0.1M HEPES buffer. Samples were centrifuged for 10 min at 14,000 rpm and the protein-containing supernatants were collected. Protein concentrations of individual samples were determined by the Bradford method (Bradford, 1976) and equimolar pools were prepared. Control protein pools were made with 92.86 µg of protein from each of the 7 samples, whereas SCZ pools were made by combining 72.22 µg of protein from each of the 9 SCZ samples. Analyses were performed in triplicate, by comparing control and SCZ protein pools.

2.4) Two-Dimensional Gel Electrophoresis

Prior to 2-DE, 650 µg of pooled proteins from SCZ or control samples were diluted to a final volume of 350 µL with a solution containing 8 M urea, 4% w/v CHAPS, 2% v/v carrier ampholytes pH 3–10, 70 mM DTT and 0.001% w/v BPB. After centrifugation at 8,000 g for 2 min, the supernatant was used for the first dimension run. Samples were applied to IPG gel strips with a nonlinear separation range of pH 3–10, 4-7 and 6-11. After

a 10 h rehydration, IEF was carried out at 20ºC, for 1 h at 500 V, for an additional hour at 1000 V and then for 10 h at 8000 V in an IPGphor apparatus (GE Healthcare, Piscataway, NJ) maintaining a limiting current of 50 μA per strip. First dimension strips were subjected to standard reduction and alkylation steps prior to second dimension electrophoresis. Strips were soaked for 10 min in a buffer containing 50 mM Tris-HCl (pH 6.8), 6 M urea, 30% v/v glycerol, 2% w/v SDS and 2% w/v DTT and for an additional 10 min in the same buffer containing 2.5% w/v iodoacetamide in place of the DTT. SDS-PAGE was performed on 12.5%T polyacrylamide gels and run on an SE-600 system connected to a Multitemp II refrigerating system (GE Healthcare). The IPG gel strips were sealed to the surface of the SDS gel using 0.5% w/v agarose. Electrophoresis was carried out for 1 h at 90 V at which time a constant current of 30 mA per gel was applied until the dye front reached the lower end of the gel. Proteins were detected by a Colloidal Coomassie staining protocol (Candiano et al, 2004) due to its superior reproducibility and sensitivity when compared with other protein staining methods (Westermeier and Marouga, 2005). All experiments were performed in triplicate.

2.5) Image Analysis – Determination of quantitative differences

ImageMaster 2D software, V 3.01 (GE Healthcare) was used for spot detection and pI/MW calibration. The observed isoelectric points (pIs) and molecular weights (MWs) of proteins were determined by comparison with known 2-D and 1-D marker proteins (Bio-Rad, Hercules, CA) that were run independently and co-migrated with DLPFC samples. MW and pI values of the markers were plotted respectively as Y and X coordinates of the 2-DE gels and equations were fitted by First-order LaGrange regression. Using these

equations, the x and y coordinates of each spot were converted to observed pI and MW values, respectively.

Significant protein expression differences between SCZ and control gels were determined using ANOVA. Protein spots with a mean n-fold change between SCZ and control DLPFC gels of +/- 1.3 were excised for identification by mass spectrometry (MS).

2.6) Protein identification by peptide mass fingerprinting

Gel spots, which showed quantitative differences by Image Master Analysis, were excised and subjected to MS-based identification. Peptides were generated and extracted from the gel-separated proteins following established in-gel trypsin digestion protocols (Shevchenko et al., 1996) using sequencing grade modified porcine trypsin from Promega (Madison, WI).

Mass spectra from each spot were acquired using an Ultraflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Measurements were performed with N_2 laser (λ: 337nm) in positive reflector mode and a 20 kV acceleration voltage. One hundred single laser shots were accumulated for each MS spectrum and 1,000 shots were accumulated for each MS/MS spectrum. Flexcontrol v.2.2 software (Bruker Daltonics) was used to acquire and process automatically MS and MS/MS data.

Acquired MS/MS spectra were searched against the NCBI database (Dec.16th, 2006) using an in-house version of MASCOT search engine 2.1 (Matrix Sciences, London, UK) embedded into the Biotools v.3.0 software (Bruker Daltonics). Parameters used were as follows: *Homo sapiens* as organism, Trypsin as enzyme allowing one missed cleavage, carbamidomethylation and oxidized methionine as fixed modifications. The mass accuracies of the precursor and fragment ions were 150 ppm and 0.7 Da, respectively.

3) Results

3.1) 2-DE of SCZ and control samples

2-DE profiles of pooled samples were generated in triplicate. On average, 750 spots were detected in each 2-DE (Figure 1). Only proteins which appeared to be differentially expressed in all three gels were considered as differentially regulated. When SCZ and control pools were compared, 22 spots (~3%) were consistently identified with significant changes in relative abundance (>1.3 fold difference, $P<0.05$, ANOVA) (Table 2). An analysis of these 22 spots revealed 24 distinct proteins with potentially altered regulation in the brains of schizophrenic patients, with 11 downregulated and 13 upregulated in SCZ samples (Table 3 and Figure 1). Sixteen proteins were identified as single spots, four proteins were identified in multiple spots (spots 8, 11, 19, 20 – Figure 1) and four proteins were identified in two spots (the spots 9 and 16 were doublets). All these 24 proteins were successfully identified by MALDI-TOF/TOF and grouped according to their functional classes using the Human Protein Reference Database (HPRD – http://www.hprd.org) (Table 3).

4) Discussion

4.1) Analysis of protein pools

A number of reasons prompted us to compare protein pools instead of individual protein extracts. While we are aware that more dramatic alterations in certain proteins of a single individual might 'contaminate' the pool, suggesting unreal alterations, we believe that the advantages of sample pooling may overcome its disadvantages. The first clear

advantage of sample-pooling is the possibility of reducing individual proteome variations (not related to the disease), while highlighting the most consistent (disease-related) alterations. Other advantages include an important reduction in the amount of protein required from each sample, allowing experimental replicates and subsequent studies. This approach has been successfully used by different authors not only for proteomics (Jiang et al. 2003; Lehmensiek et al., 2007a and 2007b), but also for gene expression analysis (Vawter et al. 2001; Katsel et al. 2005).

4.2) Confounding factors

We cannot affirm that all protein alterations detected in our pool of brain protein analysis are directly associated with SCZ. If associated with SCZ, we also can not affirm that their regulation is causative of the disease process or a consequence of the disease, age, gender, diet or the medications used by the patients. All these features can directly reflect in the proteome alterations observed here.

We should note that, according to Table I, all the SCZ samples used here came from patients that have made use of different antipsychotic drugs. This is a limitation of the present and many other studies, and should certainly have important effects on proteome alterations. As shown in Table I, three out of nine patients from our study were using haloperidol at death. Sugai et al (2004), using cDNA arrays from cynomolgus monkeys and Narayan et al., (2007), using *in situ* hybridization analysis of mice, showed that Myelin Basic Protein (MBP) is modulated upon haloperidol-treatment. An altered expression of apolipoprotein A-I in plasma of SCZ medicated patients was also found (La et al., 2007), whereas malate dehydrogenase, peroxiredoxin 3, vacuolar ATP synthase subunit beta and mitogen-activated protein kinase kinase 1 were found regulated in hippocampus of

chlorpromazine/clozapine treated rats (La et al., 2006). However, a considerable percentage of the proteins identified here have not been reported to be associated with any of the drugs used by the patients studied and many have been associated with SCZ processes largely independent of an exogenous drug effect, such as genetic linkage studies. We expected that the pooling strategy adopted here could contribute to dilute the protein alterations driven by haloperidol in 1/3 of our patients, but we cannot exclude that some of the alterations seen here could be drug-related, rather that SCZ-related.

Whereas the study of brain samples derived from psychotropic drug naïve patients is of utmost importance (such as published by Prabakaran et al. 2007), the vast majority of samples available worldwide are derived from treated patients. As the samples used in the studies were derived from patients under distinct therapeutic regimens, the recurrent identification of the same targets might implicate certain genes and proteins in the pathobiology of the disease. Thus, we believe that the data described here might add by reinforcing the importance of certain pathways, and contribute to the comprehension of this disease.

4.3) 2-DE-MS proteome analysis confirms the regulation of previously identified SCZ-related proteins and reveals new potential biomarkers

Our study revealed the alteration of some proteins previously associated with SCZ. Their identification here not only reinforces their real involvement with the pathobiology of SCZ, but it also suggests the true value of the new putative markers revealed here.

4.3.1) Oligodendrocyte metabolism:

We found in SCZ DLPFC the differential expression of myelin basic protein (MBP: downregulated: 1.5x), the major constituent of the myelin sheath in the CNS and transferrin (TF: downregulated: 2.7x), an iron carrier that participates in oligodendroglial cell differentiation, maturation and function (Espinosa de los Monteros et al., 1999; Paez et al., 2005). Both proteins were previously shown to be associated with SCZ by other groups (see Table 4 and references).

The identification of these two myelin-related proteins confirms previous studies that reported alterations of function, distribution and density of oligodendrocytes in SCZ. Approaches as diverse as diffusion tensor (Buchsbaum et al., 1998), magnetic transfer (Foong et al., 2000) or magnetic resonance imaging (Cannon et al., 1998; Wright et al., 2000; Sallet et al., 2003) allowed the observation of white matter abnormalities in SCZ, that were reinforced by gene expression (Hakak et al., 2001; Vawter et al., 2001; Tkachev et al, 2003; Prabakaran et al., 2004; Arion et al, 2007) and proteomic studies (Prabakaran et al., 2004), all confirming consistent oligodendrocyte-related alterations. While many of these alterations could be due to a reduced transcriptional activity of oligodendrocyte-related genes, there is accumulating evidence that this effect may be primarily due to a loss of oligodendrocytes or to an abnormal coherence/organization of fiber tracts that would lead to myelin-axonal disruption in SCZ brains (Hof et al., 2003; Davis et al., 2003; Kubicki et al., 2005). The reduced expression of these proteins in our SCZ samples further implicates connectivity as a central abnormality in SCZ (reviewed in Davis et al., 2003).

4.3.2) Deregulation of Energy Metabolism

We found the differential regulation of proteins that participate in glucose metabolism through glycolysis (aldolase C - ALDOC; upregulated 1.8x and phosphoglycerate kinase 1 - PGK1; upregulated 1.4x) and Krebs cycle (aconitase 2 precursor - ACO2; upregulated 1.9x and cytosolic malate dehydrogenase - MDH1; downregulated 1.3x). Moreover, we detected the differential regulation of two ATP synthase subunits (ATP5H; downregulated 1.4x and ATP5A1 – identified by 2 spots - upregulated: 2x and 1.4x) suggesting a lack in oxidative phosphorylation. Our data confirms the altered mitochondrial metabolism that has been frequently observed in SCZ (Ben-Shachar, 2002; Ben-Shachar and Laifenfeld, 2004; Middleton et al., 2002; Vawter et al., 2001; Bahn et al., 2001; Prabakaran et al., 2004). Together with other findings, there is no consensus whether these alterations are causative or consequences of a pathological process. It has also been suggested that some of the mitochondrial alterations found in SCZ could be an effect of medication (Beasley et al., 2006). However, alterations in the glucose metabolism have been extensively reported as a central component of the disease and not a simple antipsychotic effect (Stone et al., 2004). In addition, a correlation between the differential expression of glucose metabolism enzymes and oxidative phosphorylation enzymes has been recently described (Ben-Shachar et al., 2007). We also revealed the differential regulation of ubiquinol-cytochrome-c reductase complex core protein I (UQCRC1; downregulated 1.6x), never described as related in SCZ.

4.3.3) Protein metabolism:

Heat shock proteins (HSP) play numerous roles in cellular metabolism such as protein folding (Hickey et al., 1989) and mitochondrial transport (Young et al., 2003).

Whereas an abnormal immune response against heat shock 90kDa protein 1 alpha (HSPCA; downregulated 2.3x) was described in SCZ (Kim et al., 2001) some SCZ patients also present antibodies reactive to Heat-shock 60kD protein 1 (HSPD1; upregulated 2x), implicating these proteins in the pathobiology of SCZ (Kilidireas et al., 1992; Wang et al., 2003). Interestingly, the identification of these two proteins further corroborates a possible role of the immune system in SCZ (Strous and Shoenfel, 2006).

Transgenic mice of protein-L-isoaspartate (D-aspartate) O-methyltransferase (PCMT1) showed animals with abnormal cortical activities and seizures that could not be controlled by antiepileptic drugs (Kim et al., 1999), and showed synapsin I to be a major substrate of the methyltransferase. This associates PCMT1 (identified by 2 spots - upregulated: 2.6x and 2.3x) to SNC development as well as cortical and synapsis function, aspects strongly altered in SCZ. Other methyltransferases were previously implicated in SCZ pathogenesis such as catechol-O-methyltransferase that plays a role dopamine release in PFC (Gogos and Gerber, 2006).

4.3.4) Cytoskeleton-related abnormalities:

Internexin neuronal intermediate filament protein (INA; upregulated 2x), an important player in neuronal intermediate filament inclusion disease (Cairns et al., 2004), is described here for the first time as a potential SCZ marker. INA interacts with glial fibrillary acidic protein (GFAP; downregulated 1.7x), which was demonstrated to be altered in astrocytes of SCZ, bipolar disorder, and depression (Miguel-Hidalgo et al., 2000; Webster et al., 2001). INA also interacts with the neurofilament, light polypeptide (NEFL; downregulated 2.1x) that is directly involved in NMDA receptor function (Ehlers et al., 1995), in agreement with previously described glutamatergic dysfunction in SCZ (Meador-

Woodruff and Healy, 2000; Olney and Farber 1995). The glutamatergic dysfunction can modulate dynamin (DNM1 – identified by 2 spots; upregulated: 2.5x and 2.1x) levels.

Cofilin 1 (CFL1 – spot 14 – downregulated 5.3x) is an actin filament-related protein, not previously related to SCZ. CFL1 interacts with BDNF (Fass et al., 2004), a myelin basic protein expression modulator (Hohlfeld et al., 2000) previously described as regulated in SCZ (Angelucci et al., 2005; Chambers and Perrone-Bizzozero, 2004).

Crystallin mu (CRYM – spot 21 – downregulated 1.8x), another protein not previously related to SCZ, interacts with GFAP in the brain (Nicholl and Quinlan, 1994) and modulates cytoskeleton assembly (Liang and MacRae, 1997). CRYM protein was found upregulated in astrocytes associated with senile plaques and cerebral amyloid angiopathy in AD patients (Wilhelmus et al., 2006) and inhibits the aggregation of Aβ peptide *in vitro* (Wilhelmus et al., 2006 - 2).

Three cytoskeleton-related proteins that were found regulated in SCZ-DLPFC have been previously described to be altered in SCZ (Table 4).

4.3.5) Cellular Signaling:

Evidence for G-protein involvement in SCZ has been described (Catapano et al, 2006). Guanine nucleotide binding protein - beta polypeptide 1 (GNB1; upregulated 1.4x) is a G protein subunit, and dihydropyrimidinase-like 2 (DPYSL2; upregulated 1.5x) is an important player in neuronal development and may influence genetically the susceptibility to SCZ (Hong et al., 2005).

5) Author Disclosure:

Using 2-DE in combination with MS we were able to corroborate the importance of a number of proteins in the pathobiology of SCZ, and to reveal novel potential markers of this disease. Our study confirms the previously described regulation of MBP and TF, which are among the most promising SCZ biomarkers. The speculation about the role of oligodendrocyte axonic system malfunctioning in SCZ should be viewed with caution since it is not clear if the alterations seen here reflect oligodendrocyte dysfunction, a reduction in the number of oligodendrocytes in the samples or a combination of both. Besides, three out of our nine patients were using haloperidol before death. As the chronic use of haloperidol leads to a reduction in the expression of oligodendrocyte/myelin-related genes (Narayan et al, 2007) these findings must be viewed with caution. We also report alterations of proteins which may lead to an imbalance of mitochondrial metabolism since ALDOC, ATP synthase subunits and MDH1 showed to be differentially expressed in SCZ brains. One quarter of the potential SCZ biomarkers identified here have never been previously suggested, including promising candidate markers such as PCMT1 and INA whose biological function makes them attractive molecules for further studies in SCZ.

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7-) Contributors:

This work is part of the PhD thesis of DMS, who executed all the experiments and wrote the first manuscript draft. WFG and AS provided the brain samples, clinical data, and contributed to the manuscript writing. GM, EHG, MNE and GHS provided support for mass spectrometry analysis as well as the data analysis and organization. SM and JCN provided the support for 2-DE analysis as well as the data analysis and organization. CWT provided guidance for the proteome analysis. EDN supervised this work and the manuscript writing. The study was conceived by DMS and EDN. All authors contributed to and have approved the final version of this manuscript.

8-) Conflict of Interest:

All authors declare that they have no conflicts of interest.

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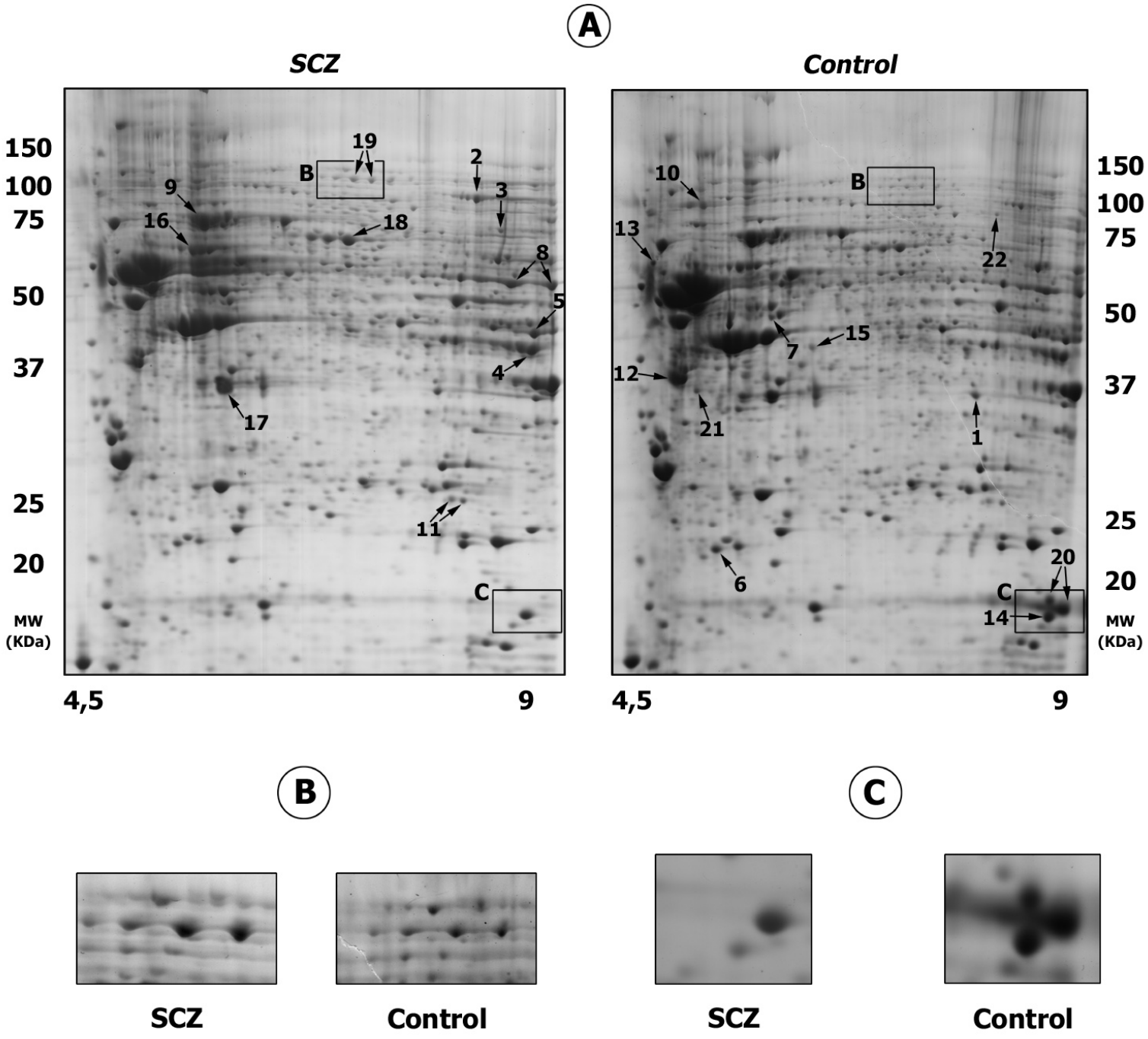

Figure 1: A) 2-DE profile of SCZ pooled samples and control pooled samples. The upregulated proteins are pointed in gels by arrows. 1B and 1C represent some detailed area of the 2-DE differential profile.

Sample ID	Case	Age (years)	Gender	PMI (hours)	Type of SCZ	Duration of Disease (years)	Duration of Medication (years)	atyptyp	CPE last dosis	CPElifetime	Cause of Death	DSM IV	Age at Onset	Last Medication	Cigarettes	Alcohol	Hosp	EKT
13/00	SCZ	64	F	11	Residual, Chronic Paranoid episodes	48	45	3	1536	7,7	pulmonary insufficiency	295.6	16	Clozapine 500 mg, Haloperidol 40 mg, Ciatyl 40 mg	0	no	21	yes
36/02	SCZ	73	M	20	Residual, Chronic Paranoid episodes	43	40	1	507,4	1,7	heart infarction	295.6	30	Perphenazine 32 mg, Promethazine 150 mg	30/day	no	33	no
39/02	SCZ	43	M	18	Residual, Chronic Paranoid episodes	22	20	2	464	2,6	heart infarction	295.6	20	Zuclopethixol 40 mg, Valproate 1200 mg, Tiapride 300 mg	0	no	13	no
39/03	SCZ	77	F	32	Residual, Chronic Paranoid episodes	49	48	2	2555	8,3	lung emboly	295.6	28	Clozapine 400 mg, Benperidol 25 mg, Chlorprothixen 150 mg	0	no	48	yes
43/03	SCZ	76	F	17	Residual, Chronic Paranoid episodes	49	47	1	300	4,9	cardio-pulmonary insufficiency	295.6	27	Perazine 300 mg	0	no	30	yes
46/00	SCZ	63	F	31	Residual, Chronic Paranoid episodes	40	30	3	75	1,8	heart infarction	295.6	24	Olanzapine 15 mg	30/day	No	30	yes
50/01	SCZ	81	M	4	Residual, Chronic Paranoid episodes	62	50	1	92,8	1,4	Cor pulmonale, heart insufficiency	295.6	19	Haloperidol 4 mg, Prothypendyl 80 mg	20/day	no	48	no
75/02	SCZ	92	F	37	Residual, Chronic Paranoid episodes	51	48	1	100	3,4	pancreas-carcinoma	295.6	41	Prothipendyl 160 mg, Perazine 100 mg	0	no	51	no
83/01	SCZ	71	M	28	Residual, Chronic Paranoid episodes	40	35	1	782,4	10	heart infarction	295.6	30	Haloperidol 32 mg, Pipamperone 40 mg	40/day	no	12	no
02/02	Control	41	M	7							heart infarction							
43/01	Control	91	F	16							cardio-pulmonary insuffiency							
50/02	Control	69	F	96							lung emboly							
51/02	Control	57	M	24							heart infarction							
57/02	Control	53	M	18							heart infarction							
59/02	Control	63	M	13							heart infarction							
61/01	Control	66	M	16							heart infarction							

Table 1: Patient and control clinical data. Abbreviations: *atyptyp:* duration of atypical treatment/duration of treatment with typical neuroleptis during lifetime; *CPE:* medication calculated in chlorpromazine equivalents (mg); *CPE last ten years:* the sum of medications during the last ten years in kg; *Hosp:* Hospitalization time in years; *ECT:* electroconvulsive therapy.

Biological Process	Reg. in SCZ	Fold Change	Spot	Accession	Protein Name	pI (th)	MW (th)	Chr Loci	Pept. Matched	MASCOT Score
Metabolism/Energy pathways	↓	-1.3301	1	gi|5174539	cytosolic malate dehydrogenase; soluble malate dehydrogenase	6.91	36426	2p13.3	12	370
	↑	1.8803	2	gi|4501867*	aconitase 2 precursor; aconitate hydratase; citrate hydro-lyase	7.36	85425	22q13.2-q13.31	29	335
	↑	4.4406	3	gi|388891	transketolase	7.89	67877	3p14.3	28	102
	↑	1.8248	4	gi|30582851	aldolase C, fructose-bisphosphate	6.41	39456	17cen-q12	25	440
	↑	1.4163	5	gi|4505763	phosphoglycerate kinase 1	8.30	44615	Xq13	47	616
	↓	-1.4301	6	gi:5453559	(ATP5H) ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d isoform a;	5.21	18491	17q25	13	213
	↓	-1.643	7	gi:92090651	Ubiquinol-cytochrome-c reductase complex core protein I, mitochondrial precursor	5.94	53270	3p21.3	17	236
	↑	-2,006 and -1,4799	8	gi:50345980	(ATP5A1) ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit isoform b	8.24	54574	18q12-q21	26	346
	↑	1.6521	9	gi|15341906	(ATP6V1A) ATPase, H+ transporting, lysosomal 70kD, V1 subunit A, isoform 1	5.35	68660	3q13.2-q13.31	23	251
Protein metabolism	↓	-2.2864	10	gi:83699649	heat shock 90kDa protein 1, alpha	5.07	98113	14q32.33	20	233
	↑	1.6521	9	gi|5729877*	heat shock 70kDa protein 8 isoform 1; heat shock cognate protein, 71-kDa; heat shock 70kd protein 1	5.37	71082	11q24.1	34	211
	↑	2.5675 and 2.3037	11	gi:14250587*	Protein-L-isoaspartate (D-aspartate) O-methyltransferase	6.7	24806	6q24-q25	9	245
	↑	2.0499	16	gi:31542947	chaperonin; mitochondrial matrix protein P1; P60 lymphocyte protein; heat shock 60kD protein 1	5.7	61187	2q33.1	25	338
Cell growth/maintenance	↓	-1.6835	12	gi|38566198	GFAP protein	5.42	49776	17q21	44	395
	↓	-2.1020	13	gi|24658018*	NEFL protein	4.64	61536	8p21	20	80
	↓	-5.3028	14	gi:30582531*	cofilin 1 (non-muscle)	8.22	18719	11q13	14	172
	↓	-1.5497	15	gi:4501887	actin, gamma 1 propeptide; actin, cytoplasmic 2;	5.31	42108	17q25	21	354
	↑	2.0499	16	gi|14249342	internexin neuronal intermediate filament protein, alpha; neurofilament-66;	5.34	55528	10q24.33	22	140
Cell communication/Signal transduction	↑	1,4278	17	gi|30583449	guanine nucleotide binding protein (G protein), beta polypeptide 1	5.6	38151	1p36.33	14	246
	↑	1.5015	18	gi:4503377*	dihydropyrimidinase-like 2; collapsin response mediator protein hCRMP-2	5.95	62711	8p22-p21	33	514
	↑	2.4971 and 2.1430	19	gi|39795292	Dynamin 1	6.57	96379	9q34	35	318
Oligodendrocyte Metabolism	↓	Absent and -1.5185	20	gi|37590005	Myelin Basic Protein	11.11	19453	18q23	22	323
	↓	-2.6981	22	gi:37747855	Transferrin	6.97	79310	3q22.1	35	288
Osmoregulation/Hormone metabolism	↓	-1.7675	21	gi|4503065	crystallin, mu; NADP-regulated thyroid-hormone binding protein	5.06	33925	16p13.11-p12.3	8	137

Table 2: Proteins regulated in Schizophrenia samples, classified according to their biological function. Proteins marked with (*) are encoded by genes that map to genomic regions previously associated with Schizophrenia. The accession numbers are from the NCBI database.

Functional class	***Total altered proteins***	***Schizophrenia***	
		Upregulated	**Downregulated**
Metabolism/Energy Pathways	9	6	3
Protein metabolism	4	3	1
Cell growth/maintenance	5	1	4
Cell communication Signal transduction	3	3	0
Oligodendrocytes Metabolism	2	0	2
Osmoregulation/Hormone metabolism	1	0	1
Total	24	13	11

Table 3: Regulated proteins in SCZ DPFC according to their functional class.

Table 4: Proteins we found regulated in DLPFC SCZ previously described by other SCZ tissues analysis. Genes/proteins marked with * were indirectly validated by those correspondent authors.

Gene symbol	Product Name	Type of analysis	Tissue	Described as regulated by:
MBP*	Myelin Basic Protein	1- immunoassay 2 - microarray and qPCR	1- anterior frontal cortex 2- pre-frontal cortex	1- Honer et al., 1999 2-Tkachev et al., 2003
TF*	Transferrin	1- microarray 2- microarray 3- microarray and proteomics 4- microarray 5- *in situ* hybridization	1- pré-frontal cortex 2- pré-frontal cortex 3- pré-frontal cortex 4- pré-frontal cortex 5- anterior cingulate córtex	1- Hakak et al., 2001 2- Tkachev et al., 2003 3- Prabakaran et al., 2004 4- Arion et al., 2007 5- McCullumsmith et al., 2007
ALDOC*	Fructose bisphosphate aldolase C	1- immunoassay* 2- proteomics 3- microarray and proteomics 4- proteomics	1- human CSF 2- frontal cortex 3- pré-frontal cortex 4- anterior cingulate cortex	1- Willson et al., 1980 2- Johnston-Wilson et al., 2000 3-Prabakaran et al., 2004 4- Clark et al., 2006
ATP5A1*	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit isoform b	qPCR	hippocampus	Altar et al., 2005
GFAP*	Glial fibrillary acidic protein	1- proteomics 2- *in situ* hybridization 3- microarray and qPCR	1- frontal cortex 2- cingulate cortex 3- pre-frontal cortex	1- Johnston-Wilson et al., 2000 2- Webster et al., 2005 3- Tkachev et al., 2003
MDH	Malate dehydrogenase	1- microarray 2- microarray 3- microarray	1- pré-frontal cortex 2- pré-frontal córtex 3- lymphocytes	1- Middleton et al., 2002 2- Vawter et al., 2004 3- Vawter et al., 2004-2
ATP6V1A	ATPase, H+ transporting, lysosomal 70kD, V1 subunit A, isoform 1	cDNA array	hippocampus	Altar et al., 2005
NEFL	Neurofilament triplet L protein	1- proteomics	1- corpus callosum	1- Sivagnanasundaram et al., 2007
DNM1	Dynamin	proteomics	anterior cingulate cortex	Clark et al., 2006
DPYSL2	Dihydropyrimidinase-like 2	proteomics	frontal cortex	Johnston-Wilson et al., 2000

Capítulo 5:

Análise Comparativa do Proteoma de Lobo Temporal Anterior Cerebral de Pacientes com Esquizofrenia e Controles Utilizando **Shotgun Proteomics**.

Através de um manuscrito submetido ao periódico "Biological Psychiatry" descrevemos os dados por nós revelados do proteoma comparativo de lobo temporal anterior de pacientes com esquizofrenia e controles por *Shotgun Proteomics*.

Proteome analysis of the anterior temporal lobe in schizophrenia reveals alterations in oligodendrocyte proteins and in calcium homeostasis

Daniel Martins de Souza[1,2,3], Wagner F. Gattaz[1], Andrea Schmitt[4,5], Christiane Rewerts[2], Sérgio Marangoni[3], José C. Novello[3], Giuseppina Maccarrone[2], Emmanuel Dias-Neto[1,6,7] & Christoph W. Turck[2,7].

1- Laboratório de Neurociências – Instituto de Psiquiatria – Faculdade de Medicina da USP – São Paulo – SP – Brazil
2- Max Planck Institute of Psychiatry - Kraepelinstrasse 2, D-80804 – Munich - Germany
3- Laboratório de Proteômica – Departamento de Bioquímica - Instituto de Biologia – UNICAMP – Campinas – SP - Brazil
4- Department of Psychiatry, Von Siebold Str. 5, University of Goettingen, 37075 Goettingen, Germany
5- Central Institute of Mental Health, J5, 68159 Mannheim, Germany
6- Present address: University of Texas, MD Anderson Cancer Center, 1515 Holcombe Blvd, 77030, Houston, Texas, USA.

7 - Corresponding Authors (these authors contributed equally to this work):
Christoph W. Turck
Phone: 49-89-30622-317
Fax: 49-89-30622-610
turck@mpipsykl.mpg.de

Emmanuel Dias-Neto
Phone +1 713 794.5911
emmanuel@usp.br
edneto@mdanderson.org

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Abstract

Global proteomic analysis of post-mortem anterior temporal lobe samples from schizophrenia patients and non-schizophrenia individuals was performed using stable isotope labeling and shotgun proteomics.

Our analysis resulted in the identification of 479 proteins, forty-five of which showed statistically significant differential expression. Pathways affected by differential protein expression include transport, signal transduction, energy, cell growth and maintenance, protein metabolism, regulation of DNA and RNA metabolism and immune response.

The collection of protein alterations identified here reinforces the importance of myelin/oligodendrocyte and calcium homeostasis in schizophrenia, and reveals a number of new potential markers that may contribute to the understanding of the pathogenesis of this complex disease.

Introduction

Schizophrenia (SCZ) is an exclusive human pathology comprising a wide range of neurodevelopmental, neurophysiological, neurochemical, and psychological disturbances influenced by genetic components and environmental factors, which presents a wide range of positive and negative symptoms (Gattaz and Haffner, 2004). In brain, there are patterns of synchronization and desynchronization which communicate brain areas through specific neuronal activity (Singer 1999). In a complex disease as SCZ it is possible that all brain areas have a potential role since they are all connected. However, there are some areas which seem to be more involved based on their exerted functions. The pre-frontal cortex because of its executive functions (Miller and Cohen, 2001), the basolateral amygdala because of its expression of affective behavior (Davis and Whalen, 2001), and the anterior cingulate cortex because of its expression of cognitive and affective processes (Carter et al., 1997).

The temporal lobe concentrates important functions such as high-level auditory and visual processing, language, and transference from short to long-term memory. All these functions can be compromised in SCZ in a process that apparently involves an imbalance of glutamate and GABA leading to dopaminergic dysfunctions (Deakin and Simpson, 1997). A reduction of the size of the temporal lobe in SCZ has been shown by several studies with magnetic resonance imaging (MRI) (Bogerts 1993; Suddath et al, 1998). In the temporal cortex of SCZ patients, deficits were reported in glutamate presynaptic components (Deakin and Simpson, 1997); glutamate uptake sites were reduced on the left side (Deakin et al, 1989) with no losses of post-synaptic glutamate receptors (Nishikawa, 1983). Deakin

and Simpson (1997) have shown that degenerated glutamate terminals in the temporal lobe originate in the frontal cortex with important implications for SCZ.

In the present work we performed a quantitative proteomic analysis of the Anterior Temporal Lobe (ATL) of SCZ and control samples using Isotope-Coded Protein Label (ICPL), a method for the accurate quantitative comparative analysis of protein regulation (Schmidt et al, 2005). ICPL is based on isotope labeling of free amino groups in intact proteins. After the modification, the heavy and light isotope labeled proteins are digested and analyzed by liquid chromatography (LC) followed by tandem mass spectrometry (MS/MS). Relative quantification of differential protein expression is based on the comparison of the peak intensities of the heavy- and light-labeled peptides from the mass spectra. The ICPL method is very reproducible and compatible with all known protein and peptide separation techniques, providing highly accurate quantification of regulated proteins.

The identification of proteins differentially expressed in the ATL of SCZ patients was performed by MS/MS followed by subsequent database searches. After their validation in a large set of samples and patients, these proteins can be potentially valuable not only for a better understanding of the biological basis of the disease, but also as biomarkers for disease monitoring or as targets for pharmaceutical applications.

Materials and Methods

Materials

All chemicals and solvents were from Bio-Rad (Hercules, CA, USA) and of the highest purity available. The ICPL kit was from Serva Electrophoresis (Heidelberg,

Germany) and Prespotted AnchorChips were obtained from Bruker Daltonics (Bremen, Germany).

Human Anterior Temporal Lobe samples

Frozen tissue blocks from the left anterior temporal lobe tissue, Brodmann Area (BA) 22, were collected *post-mortem* from five SCZ patients and four controls free of psychiatric disorders. The left side was selected due to its importance in SCZ (DeLisi et al, 1989).

All brain samples were obtained from Central Institute of Mental Health (Mannheim, Germany), dissected by an experienced neuropathologist and deep-frozen in liquid nitrogen-cooled isopentane immediately after dissection. On average, the samples were collected 17.8 h (minimum: 4 h / maximum 28 h) after death. All cases were Caucasian and German with one female in each group. Detailed patient data are listed in Table 1. All SCZ patients had been long-term inpatients at the Mental State Hospital Wiesloch, Germany. Patients who died had been diagnosed *ante mortem* by an experienced psychiatrist according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders IV (DSM IV) of the American Psychiatry Association for Schizophrenia (American PA, 1994). For each patient the history of antipsychotic treatment was assessed by examining the medical charts and calculated in chlorpromazine equivalents (CPE). All assessments and *post mortem* evaluations and procedures had been approved by the Ethics Committee of the Faculty of Medicine of Heidelberg University, Germany.

All patients and controls underwent thorough neuropathologic characterization to rule out associated neurovascular or neurodegenerative disorders such as Alzheimer's disease and multi-infarct dementia (Braak and Braak 1991, Braak et al. 2006). The staging

according to Braak was stage 2 or less for all subjects. Patients and normal comparison subjects had no history of alcohol or drug abuse, or severe physical illness.

Sample Preparation

Fifty milligrams of human temporal lobe tissue from control and SCZ subjects were grind in 1.5ml tubes, together with glass spheres, in 200µl of 6M guanidine and 0.1M HEPES buffer. Protein concentrations were determined using the Bradford assay (Bradford, 1976). Brain proteins were evaluated in pools containing 100µg of protein. The SCZ pool was prepared by combining 20µg of proteins obtained from each of the five SCZ samples, whereas the control pool contained 25µg of each of the four controls. Pooled samples were used in order to reduce individual variability not related to the investigated phenotype (Weinkauf et al., 2006).

ICPL labeling

One hundred micrograms of total protein from SCZ or controls (5 mg/ml) were reduced, as specified by the ICPL kit protocol, for 30 min at 60ºC. After cooling to RT, free thiol groups were alkylated in the dark with 1 ml of 0.4 M iodoacetamide for 30 min at RT. Excess iodoacetamide was quenched by adding 1 ml of 0.5 M N-acetylcysteine. For protein labeling a ten-fold molar excess (based on free amino groups) of light tag for the control sample and heavy tag for the SCZ samples were added to the proteins and the reactions allowed to proceed for 2 h at RT. Four ml of 1.5 M hydroxylamine were added to each sample to inactivate the remaining Nic-NHS reagents, and equal aliquots of both samples

were combined. Esters, which are also formed during the labeling procedure, were hydrolyzed by raising the pH to 11–12 for 20 min.

Digestion of labeled proteins and fractionation of peptides by isoelectric focusing

Protein samples were digested in 200 mM NH_4HCO_3 pH 8.3 with 1mg/ml trypsin at a ratio 1:50 (P:E) at 37° C for 4 h. Resultant peptides were fractionated on Immobilized pH gradient strips (IPG 17cm), pH 3.5-4.5. The strips were rehydratated for 12 h and run for 8 h with a constant voltage of 10,000V. The strip was manually cut in 47 pieces and the peptides extracted with 1% formic acid.

Fractionation of peptides by nano high performance liquid chromatography

Each of the 47 peptide samples from isoelectric focusing was further fractionated on a micro-LC-System (HP1100 Agilent Technologies, Waldbronn, Germay) using an RP-C-18 monolithic column (200µm id. x 5cm, Dionex, Sunnyvale, CA) with a flow rate of 4 µl/min and a 40 min gradient from 10 to 100 % of solvent B (ACN; 0.1% TFA). Each isoelectric focusing eluate was chromatographed and fractions were collected onto Prespotted AnchorChip targets (Bruker Daltonics) and providing matrix thin layer preparations using a PROTEINEER-FC robot (Bruker Daltonics).

Mass spectrometry

Mass spectra from each target spot were acquired fully automatically using an Ultraflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics). Measurements were performed with a nitrogen laser in positive reflector mode and a 20,000 V acceleration voltage. For MS spectra 100 shots and for MS/MS spectra 1,000 shots were accumulated.

WARP-LC 1.0 software was used for spectra acquisition and controlling the automatic selection of peptides for further MS/MS analysis. The ICPL–labeled peptides were selected for the MS/MS analysis based on their H/L ratio.

Identification of Proteins

Acquired MS and MS/MS spectra were automatically sent as combined peak lists by the WARP-LC 1.0 to Biotools software 3.0 (Bruker Daltonics) and searched against the NCBI database (Dec.16th, 2006) using an in-house version of MASCOT 2.1 (Matrix Science, London, UK). Parameter settings: *Homo sapiens* for organism, trypsin and Arg-C for enzymes, carbamidomethylation as fixed modification and oxidized methionine and heavy and light ICPL labels of lysines and N-terminal protein as variable modifications.

ICPL Quantitative Analysis

The determination of the ratios of isotope-labeled peptide pairs (heavy and light) was performed by WARP-LC 1.0 Protein Browser (Bruker Daltonics), comparing the relative heavy and light cluster signal intensities. The identified heavy and light peptide-pair sequences containing up to four labeled lysines with a mass difference of 6.0204 Da per labeled amino group were obtained by BioTools 3.0. The workflow of protein shotgun mass spectrometry and ICPL-quantitation of differentially expressed proteins is shown in Figure 1.

Determination of regulated proteins in SCZ samples

Three parameters were applied to determine the putative regulated proteins.

1) BioTools software returns for each identified protein a MASCOT score value that is derived from the peptide hit scores. Only peptide scores greater than 38 were considered significant identifications.

2) The regulation status of a protein was defined by the ratio of the relative peptide signal intensities. We labeled SCZ proteins with heavy tag and control proteins with light tag. The proteins in both samples generated the same peptides after digestion, but peptides from SCZ samples had an approximately 6 Da per labeled amino group greater mass than peptides labeled with light tags (Figure 2). In our analysis we considered a minimum 40% variation as significant regulation. Thus, proteins upregulated in SCZ should have ratios ≥1.4, whereas downregulated proteins should have ratios ≤0.6. Ratio values of 0.6 – 0.8 or 1.2 – 1.4 were considered borderline.

3) We analyzed the identity of the protein and its function using the Human Protein Reference Database (HPRD) based on Gene Ontology criteria.

Results

Protein regulation in Schizophrenic anterior temporal lobe

Shotgun mass spectrometry resulted in the analysis of 837 peptide sequences (Figure 2A) and lead to the identification of 479 proteins in ATL (Figure 2B); 269 proteins (56.2%) were identified by unlabeled peptides and 210 (43.8%) by labeled peptides.

For relative quantification, peptide data for these 210 proteins were evaluated. No significant variations between SCZ and control samples were observed for 165 proteins (78.6%). Of the remaining 45 proteins, 10 (22%) appeared to be upregulated and 35 (78%) downregulated. All proteins that were regulated in the ATL of SCZ patients could be

unambiguously identified and are listed in Table 2. One of the upregulated and 11 of the downregulated proteins are products of genes that are part of genomic loci which have been previously associated with SCZ.

Functional classification of regulated proteins

The regulated proteins were divided in functional classes according to the Human Protein Reference Database (HPRD – http://www.hprd.org) and are shown in Table 3. Most of them belong to cell communication and signal transduction (11/45), cell growth maintenance (10/45) and energy metabolism (9/45) pathways. Transport is the only category in which upregulation overcomes downregulation in SCZ ATL brains.

Discussion

ICPL methodology for Proteomic Analysis

For our analysis, we employed shotgun proteomics to overcome some of the limitations of conventional proteomic strategies such as two-dimensional gel electrophoresis. This allowed us to investigate a greater fraction of the proteome and to more accurately measure differences in protein expression. The quantification of shotgun-generated data is improved by the use of stable isotope labeling of the proteins and allows a more precise comparison and quantification (Schmidt et al, 2005). This approach was applied for pooled control and SCZ samples, in order to identify proteins with altered regulation in the disease. Most of the regulated proteins identified (84.4%) were found to be reduced in SCZ ATL. The overall reduction in protein translation could be a hallmark of the hypotemporality described in SCZ (Catafau et al, 1994).

Shotgun proteomics confirms the regulation of previously identified SCZ-related proteins

Oligodendrocyte-myelin regulated proteins:

The most important function of the oligodendroglia is the myelination and maintenance of myelin sheets in axons of the central nervous system (CNS). The diminution or malformation of the myelin sheath results in an increased ion leakage and a reduced propagation of nerve impulses. Besides, other functions as trophic signalling to nearby neurons, synthesis of growth factors, neuronal survival and development, neurotransmission and synaptic function are executed by oligodendrocytes (Du and Dreyfus, 2002; Deng et al., 2003).

Whereas an analysis by magnetic transfer imaging and diffusion tensor imaging showed a disruption of white-matter integrity in SCZ patients (reviewed in Segal et al., 2007), several cDNA microarray studies (Hakak et al., 2001; Tkachev et al., 2003; Aston et al., 2004; Katsel et al., 2005) and individual gene expression analysis (Webster et al., 2005; Dracheva et al., 2006; McCullumsmith et al., 2007) revealed the alteration of a series of myelin-related genes in SCZ. A set of proteins encoded by these genes were also identified in our study, including 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP – downregulated here: -1.96x), glial fibrillary acidic protein (GFAP – downregulated here: -1.7x), myelin oligodendrocyte glycoprotein (MOG – downregulated here: -2.32x).

Myelin Basic Protein (MBP – downregulated here: -2.56x), the major constituent of the myelin sheath in the CNS, has a neuroprotective role *in* vivo (Moalem et al., 1999) and its expression is controlled by brain-derived neurotrophic factor (BDNF) (Hohlfeld et al., 2000) whose mRNA and protein were found regulated in SCZ patients' brain and serum (Angelucci et al., 2005; Chambers and Perrone-Bizzozero, 2004). The myelinating

oligodendrocyte-specific protein, Ermin (downregulated here: -3.23x) is a protein that seems to be exclusively expressed by oligodendrocytes. It regulates cytoskeletal rearrangements during myelinogenesis and is also important in the maintenance and stability of the myelin sheath in the adult brain (Brockschnieder et al., 2006).

The downregulation of the above mentioned proteins, revealed by an approach never seen over an independent sample set of a different brain area, strongly reinforces the importance of oligondendrocyte homeostasis in the pathogenesis of SCZ.

Regulation of Ca^{2+} homeostasis:

The identification here of an altered regulation of 5 proteins related with Ca^{+2} homeostasis and metabolism reinforces the concept of the centrality of Ca^{+2} in SCZ. Abnormal brain Ca^{2+} concentrations, probably mediated by an altered regulation of Calmodulin (CALM - downregulated here: -5x), an intracellular Ca^{2+} sensor, and by the plasma membrane calcium-transporting ATPase 4" (PMCA-4 – upregulated here: 1.59x), which is involved in the maintenance of Ca^{2+} homeostasis in the cell (Strehler and Treiman, 2004), may increase Ca^{2+}-dependent phospholipase A2 (PLA2) activity that may account for the accelerated phospholipid turnover and for reduced dopaminergic activity seen in the SCZ frontal lobe (Gattaz et al., 1990; Gattaz and Brunner, 1996).

Ca^{+2} is considered a pivotal metabolite for the dopamine hypothesis in SCZ and plays a crucial role in the function of dopamine receptors D1 and D2 (Bergson et al., 2003). It should be noted that Calcineurin, which appeared to be downregulated here (-1.67x) as well as in other SCZ studies (Hakak et al., 2001; Eastwood et al., 2005), play roles in neuronal function (Malenka, 1994; Liu et al., 1994) and is a regulator of dopaminergic (Greengard, 2001) and glutamatergic (Zeng et al., 2001) neurotransmission, which are

frequently compromised in SCZ (Seeman, 1987; Carlsson et al., 2001). Dopaminergic hyperactivity in SCZ may result in altered NMDA receptor activation, which can lead to excitotoxicity and excess Ca^{+2} influxes through NMDA receptors (Lee et al., 1999).

The altered regulation of PMCA-4 and MBP support the findings of Fu et al. (2007) which show myelin degradation by cytosolic PLA2 induced by lysophosphatidylcholine (lyso-PtdCho) via Ca^{2+} influx into myelin. We submit that PMCA-4 upregulation promotes a higher Ca^{+2} influx, leading to a stimulation of Ca^{2+}-dependent PLA2 (Gattaz et al., 1990), which increases lyso-PrdCho (Pangerl et al., 1991) which finally leads to myelin degradation.

Visinin-like protein 1 (VILIP-1 - downregulated: -1.96x) that has Ca^{+2}-dependent modulatory effects on signaling (Polymeropoulos et al., 1995) and Spectrin alpha chain (also known as fodrin - downregulated: -2.32x) that has a proteolytic activity is initiated by calcium-activated proteases, were other 2 proteins we found regulated in ATL SCZ.

Energy metabolism

Transcriptome and proteome alterations in energy metabolism have been extensively described in patients with SCZ (Prabakaran et al. 2004, Ben-Shachar and Laifenfeld 2004, Bubber et al. 2004, Karry et al. 2004, Clark et al. 2006, Martorell et al. 2006, Mehler-Wex et al. 2006) and our data confirmed these previous finding. We found the downregulation of several members of the NADH:ubiquinone oxidoreductase complex NDUFS3, NDUFS6, NDUFB5 and NDUFV2 (-1.89x, -1.72x, -2.08x and -2.08x respectively). The connection of energy metabolism in neuronal plasticity and synapse (reviewed in Ben-Shachar and Laifenfeld 2004) as well as evidences of oxidative damage in SCZ brains (reviewed in Yao et al. 2001) and dopamine toxicity through mitochondrial

complex I inhibition (Ben-Shachar et al., 2004) suggests an important energetic component in SCZ.

Other regulated proteins

In our analysis we found a number of regulated proteins that have been previously reported to be altered in SCZ (Table 4), such as hemoglobin subunit beta (-1.75x), which can compromise neuronal microcirculation (Nakashima et al., 1996) and result in attention deficits (Toichi et al., 2004) and Aquaporin type 4 (AQP4 – upregulated: 1.87) that controls the water flow within the CNS (Nagelhus et al., 1999; Wen et al., 1999) and may compromise ionic homeostasis (Fatemi et al., 2005).

The neurofilaments M (NEFM - downregulated: -2.17x) and L (NEFL - downregulated: -3.13x) that belong to a family of proteins recently named DRIP (dopamine receptor interacting protein) and have important functions in the dopamine receptor signal transduction pathway (Bergson et al., 2003); NEFL is directly associated with NMDA receptors (Ehlers et al., 1995).

New potential SCZ markers

Next to proteins that had been previously associated with SCZ pathogenesis we also found eleven proteins that had not been described previously to be regulated in ATL-SCZ. Significant differences in expression were seen for these proteins and suggest their potential role in the disease.

Prohibitin (PHB – upregulated: 1.57x) has many roles, being involved in apoptosis (Bruneel et al., 2005), maintenance of mitochondrial function and protection against senescence (Arnold and Langer, 2002). Phosphatidylethanolamine-binding protein 1

(PEBP1 – upregulated: 2.86), is a substrate of Calpain (Chen et al., 2006), a Ca^{+2}-dependent protease implicated in synaptic chemistry and structure (Etienne and Baudry, 1987) with functions in membrane biogenesis (Moore et al., 1996) which reinforce the importance of membrane phospholipid metabolism in SCZ. We should observe the recent publication of the PEBP1-knockout mouse, which revealed the role of this protein in the control of emotions and complex behavior responses (Theroux et al., 2007). The loss of PEBP1 function has been implicated in AD and behavioral testing in mice revealed a learning deficit (George et al., 2006).

We also found the downregulation of the aggregan core protein (AGC, -2x), a proteoglycan that regulates neurite growth (Ruoslahti., 1989) and contains a hyaluronic acid (HA) binding domain, which may associate this protein to the hyaluronan and proteoglycan link protein 2 (HAPLN2) that was also downregulated here (-6.25x). HA is of critical importance for the formation of brain extracellular matrix, which maintains the brain shape and correct functioning.

Validation of differentially expressed proteins

Whereas we had limitations to perform experiments to validate the potential markers identified by our shotgun approach, we could observe that most of the markers seen here have been previously identified or validated by other groups. Such recurrent genes/proteins (marked with an asterisk in Table 4), gives us confidence that the repetitive findings are true disease identifiers, and that the new markers found here are potentially related to the disease.

Confounding factors: the ATL-SCZ proteome and the effect of antipsychotics:

At this point we do not know whether the alterations detected in our analysis are a cause or a consequence of the disease. We also cant discard that these markers arised from a combination of confounding factors such as age, smoking, duration of disease (Table 1), circadian rhythm variations, physical exercise, food intake or medication. All these features can potentially contribute to proteomic alterations.

The brain samples used here were derived from SCZ patients taking varying doses of different neuroleptics (Table 1). Two of the five patients in our study were using haloperidol just before their death. Sugai et al (2004), using cDNA arrays from cynomolgus monkeys and Narayan et al., (2007), using *in situ* hybridization in mice, showed that MBP can be modulated by haloperidol-treatment. It was also reported that apolipoprotein A-I expression in plasma of SCZ medicated patients is altered (La et al. 2007). Malate dehydrogenase, peroxiredoxin 3, vacuolar ATP synthase subunit beta and mitogen-activated protein kinase kinase 1 were found regulated in the hippocampus of chlorpromazine/clozapine treated rats (La et al., 2006). However, a great number of the identified proteins found to be regulated in the present study have been reported to be associated with SCZ either through genetic linkage or transcriptomic regulation, which are processes largely independent of an exogenous drug effect.

Thus, we can not affirm which proteins among the 45 described here are modulated by antipsychotic medication. Despite the Narayan et al. (2007) findings, we can not affirm that the oligodendrocyte-related proteins found here are consequence of antipsychotic use and even affirm categorically that these proteins are SCZ agents. In future studies the characterization of the proteome from naïve tissues will show whether the identified differences are SCZ pathogenesis-related or due to other confounding factors.

Conclusion:

The comparison of protein profiles of ATL from SCZ and controls by shotgun proteomics enabled the identification of proteins differentially expressed in diseased samples. Whereas gene polymorphisms and gene expression alterations can play an important role in SCZ, the identification of disease-related proteins, the true biological effectors, is critical for the understanding of the pathophysiology of the disease.

We do not know whether the identified proteins whose expression is regulated in SCZ are causative for or a consequence of the disease or might be a reflection of the medications given to the patients. However, the identification of proteins that are part of pathways previously reported to be associated with the disease indicates some kind of involvement in the pathology of SCZ.

The observation that many of the markers identified in our analysis have been previously revealed by other groups using alternative approaches such as cDNA microarrays, reinforces their association with SCZ. Together with other studies, our findings suggest the involvement of oligodendrocytes and myelin dysfunction as well as calcium and energy imbalance in SCZ. A central concept of SCZ neurobiology is that its symptoms may arise from a malfunctioning communication between different brain areas, leading to a disruption of the circuitry that underlies behavior and perception. In this context, structural and functional abnormalities leading to a brain dysfunction may comprise not only neurodevelopmental and neurotransmitter aspects, but may also include factors important for impulse propagation such as intracellular Ca2+ and axonal insulation.

Whereas it is tempting to speculate that a decomposition of the oligodendrocyte-axonic system may be responsible for some SCZ symptoms, the results should still be

viewed with caution. It is not clear if the alterations seen here reflect oligodendrocyte dysfunction, a reduction in the number of oligodendrocytes in the samples or a combination of both, or the simple use of haloperidol by two out of our five patients.

The regulation of Ca^{2+} homeostasis-related proteins reinforces the importance of this pathway in SCZ. Ca^{2+} is of pivotal importance for dopamine receptor function (Bergson et al, 2003) and studies of genetic association have revealed calcium-activated potassium-channels as important players in SCZ (Chandy et al, 1998). Moreover, Ca2+ influences the activity of several enzymes related to neuronal membrane function, such as a group of phospholipases A2, which consistently have been shown to be altered in schizophrenia (Gattaz et al 1987, Barbosa et al, 2007). Mitochondrial dysfunction in SCZ is also indicated by the downregulation of several proteins from mitochondrial complex I. In addition, we have identified many proteins that have not previously been associated with SCZ pathogenesis. These include PEBP1 which was recently identified as an important protein involved in complex behavior responses in the brain (Theroux et al. 2007).

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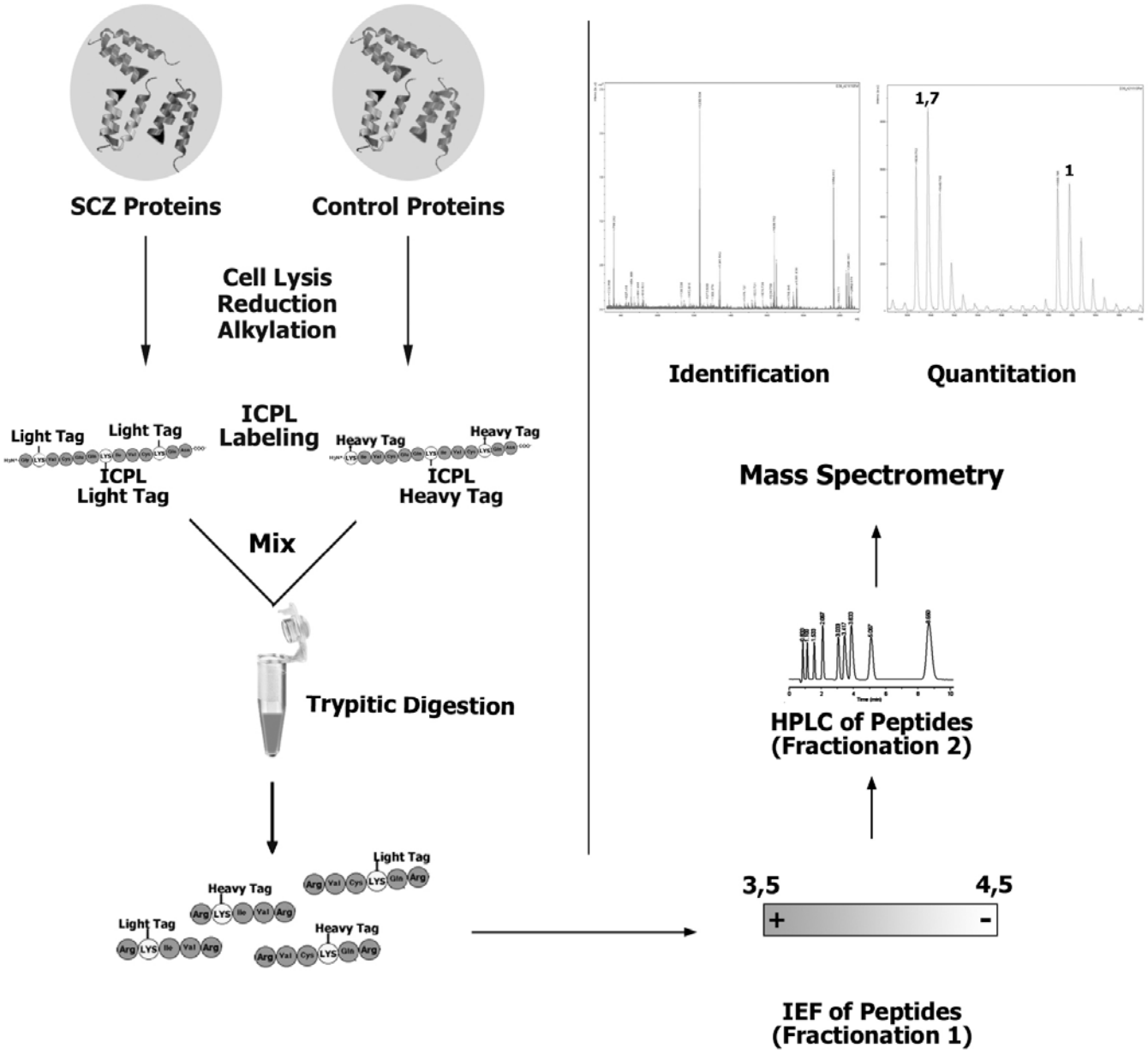
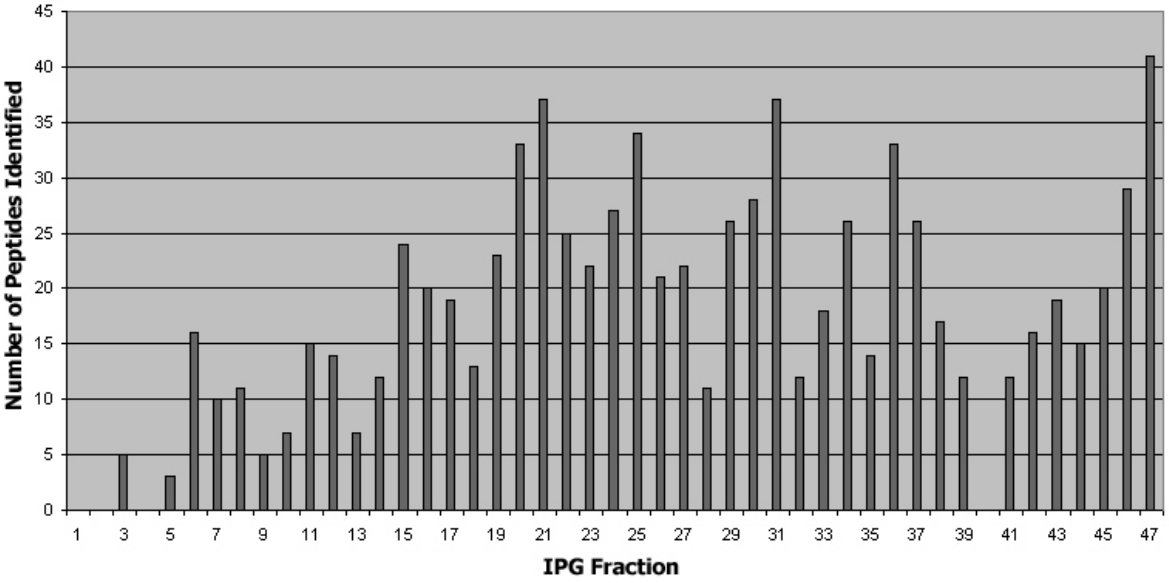


Figure 1: Proteomic workflow: after cell lysis proteins are labeled with light or heavy ICPL reagents, combined and digested with trypsin. Tryptic peptides are fractionated by isoelectric focusing (IEF) on an IPG strip and then subjected to LC-MALDI mass spectrometry for identification and quantitation.

A



B

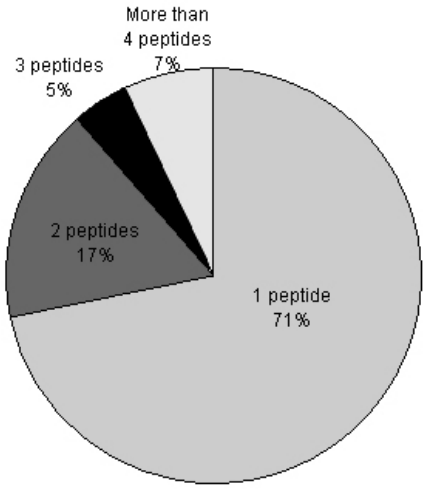


Figure 2: Shotgun mass spectrometry results. A) Number of peptides identified from each IPG fraction; B) Number of peptides that identified a protein.

Sample ID	Case	Age (years)	Gender	PMI (hours)	pH values	Type of SCZ	Duration of Disease (years)	Duration of Medication (years)	atyptyp	CPE last dosis	CPE last ten years	Cause of Death	DSM IV	Age at Onset	Last Medication	Cigarettes	Alcohol	Hosp	ECT
01/00	SCZ	51	M	12	6,7	Residual, Chronic Paranoid episodes	28	25	2	450	1.8	heart infarction	295.6	23	Clozapine 500 mg	30/day	no	17	No
35/00	SCZ	64	F	23	6,6	Residual, Chronic Paranoid episodes	41	40	2	54.5	4.6	heart infarction	295.6	24	Zotepine 150 mg Olanzapine 10 mg	20/day	no	5	yes
36/02	SCZ	73	M	20	6,9	Residual, Chronic Paranoid episodes	43	40	1	507.4	1.7	heart infarction	295.6	30	Perphenazine 32 mg Promethazine 150 mg	30/day	no	33	no
83/01	SCZ	71	M	28	6,5	Residual, Chronic Paranoid episodes	40	35	1	782.4	10	heart infarction	295.6	30	Haloperidol 32 mg Pipamperone 40 mg	40/day	no	12	no
50/01	SCZ	81	M	4	6,8	Residual, Chronic Paranoid episodes	62	50	1	92.8	1.4	Cor pulmonale, heart insufficiency	295.6	19	Haloperidol 4 mg Prothypendyl 80 mg	20/day	no	48	no
43/01	Control	91	F	16	6,8							cardio-pulmonary insuffiency				no	no		
57/02	Control	53	M	18	7,1							heart infarction				20/day	no		
61/01	Control	66	M	16	6,9							heart infarction				10/day	no		
72/02	Control	79	M	24	6,4							heart infarction				no	no		

Table 1: Patient and control clinical data. Abbreviations: *atyptyp:* duration of atypical treatment/duration of treatment with typical neuroleptics during lifetime; *CPE:* medication calculated in chlorpromazine equivalents (mg); *CPE last ten years:* the sum of medications during the last ten years in kg; *Hosp:* Hospitalization time in years; *ECT:* electroconvulsive therapy

Biological Process	Regulation in SCZ	Protein Name	Accession	H/L Ratio	pI (th)	MW (th)	Chr Loci	Id. Pept	MASCOT Score
Transport	↑	Aquaporin type4	Q6L7A0	1.87	6.20	23623.16	18q11.2-q12.1	1	50.18
	↑	Plasma membrane calcium-transporting ATPase 4	P23634	1.59	6.19	137920.16	1q25-q32	3	76.49
	↓	Hemoglobin subunit beta	P68871	0.57	6.81	15867.22	11p15.4-p15.5	1	41.1
Signal transduction; Cell communication	↑	Ezrin-radixin-moesin-binding phosphoprotein 50	O14745	1.58	5.55	38737.24	17q25.2	2	50.76
	↑	Prohibitin or PHB protein	Q6FHP5	1.57	5.57	29832.16	17q21	2	62.38
	↑	Phosphatidylethanolamine-binding protein 1	P30086	2.86	7.43	20925.59	12q24.23	3	44.07
	↓	14-3-3 protein eta	Q04917*	0.49	4.76	28087.53	22q12-q13	1	77.29
	↓	Dual specificity protein phosphatase 3	Q5RD73	0.48	7.66	20478.29	17q21	1	41.61
	↓	14-3-3 protein zeta/delta	P63104	0.52	4.73	27745.1	2p25.2-p25.1	4	58.22
	↓	Calcineurin or Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform	Q08209	0.60	5.58	58687.85	4q21-q24	3	64.34
	↓	Visinin-like protein 1	P62763	0.51	5.01	22011.14	2p24.3	3	91.89
	↓	Signal-regulatory protein beta-1	O00241	0.56	6.06	43255.18	20p13	4	118.36
	↓	14-3-3 protein gamma	P61981	0.46	4.8	28171.4	7q11.23	4	112.84
	↓	Calmodulin	P62158	0.20	4.09	16706.39	14q24-q31	8	131.36
Metabolism; Energy pathways	↑	Hexokinase brain form	P19367	1.84	6.44	102484.91	10q22	2	43.49
	↓	NDUFS6 or NADH-ubiquinone oxidoreductase 13 kDa-A subunit	O75380	0.58	8.58	13711.61	5p15.33	1	100.91
	↓	Carbonic anhydrase 2	P00918	0.50	6.86	29114.86	8q22	1	85.87
	↓	NDUFB5 or NADH-ubiquinone oxidoreductase SGDH subunit	O43674	0.48	9.62	21750.27	3q26.33	1	52.24
	↓	Succinyl-CoA ligase - mitochondrial	Q9P2R7	0.53	7.05	50317.26	13q12.2	1	89.19
	↓	NDUFV2 protein	Q6IB76	0.48	8.22	27349.47	18p11.31-p11.2	2	112.02
	↓	NDUFS3 or NADH-ubiquinone oxidoreductase 30 kDa subunit	O75489	0.53	6.98	30241.53	11p11.11	4	81.08
	↓	fructose bisphosphate aldolase C or ALDOC protein	Q6FH94	0.56	6.41	39455.87	17cen-q12	5	79.21
	↓	2',3'-cyclic-nucleotide 3'-phosphodiesterase	P09543	0.51	9.17	47578.63	17q21	13	87.29
Cell Growth and/or maintenance	↑	Tubulin, beta polypeptide	Q9BVA1*	1.46	4.78	49953.06	*6p25*	1	81.31
	↓	Lamin A/C	Q5I6Y4*	0.60	6.44	74070.38	1q21.2-q21.3	1	52.12
	↓	Aggrecan core protein	P16112*	0.50	4.1	250193.19	15q26.1	1	65.66

Cell Growth and/or maintenance	↓	Spectrin alpha chain, also know as Fodrin or Spectrin, non-erythroid alpha chain	Q13813	0.43	5.22	284539.36	9q33-q34	1	55.93
	↓	Destrin	P60981	0.56	8.12	18374.53	20p12.1	1	53.86
	↓	Glial fibrillary acidic protein, astrocyte	P14136	0.59	5.42	49880.21	17q21	16	103.48
	↓	Neurofilament triplet M protein	P07197*	0.46	4.9	102316.8	8p21	1	72.91
	↓	Hyaluronan and proteoglycan link protein 2	Q5T3J1*	0.16	8.37	20827.82	1q23.1	1	105.92
	↓	Neurofilament triplet L protein	P07196*	0.32	4.64	61385.41	8p21	2	70.24
	↓	Clathrin light chain B	P09497*	0.50	4.57	25190.49	5q35	3	74.98
Protein Metabolism	↓	Ubiquitin-conjugating enzyme E2 N	P61088	0.32	6.13	17137.82	12q21.33	1	88.98
	↓	Eukaryotic initiation factor 4A-II	Q14240	0.52	5.33	46402.27	3q28	2	46.03
	↓	60S ribosomal protein L13	P26373	0.46	11.65	24130.28	16q24.3	2	62.73
	↓	40S ribosomal protein S10	P46783*	0.53	10.15	18897.77	6p21.31	2	54.19
	↓	Endoplasmic reticulum protein or ERP29	P30040	0.38	6.77	28993.43	12q24.13	1	79.53
	↓	Ubiquitin	P62988*	0.52	6.56	8564.84	17p11.1-17p12	1	41.16
Nucleic acid metabolism	↓	Histone H3.1	P68431*	0.31	11.13	15272.89	6p21.3	2	58.35
	↓	Ribonuclease/angiogenin inhibitor 1	P13489	0.59	4.71	49842.28	11p15.5	1	83.02
Immune Response *Neurogenesis* *Synaptic Transmission* *Oligodendrocyte Metabolism*	↓	Myelin oligodendrocyte glycoprotein	Q5SSB7*	0.43	8.29	23235.99	6p22.1	1	95.48
	↓	Myelin basic protein	Q65ZS4	0.39	11.35	22316.06	18q22-qter	7	78.43
	↓	Ermin (myelinating oligodendrocyte-specific protein)	Q8TAM6	0.31	4.75	32782.95	2q24.1	2	61.42
Biological process unknown									
	↓	SH3-domain GRB2-like endophilin B2	Q9NR46	0.52	5.72	43973.94	9q34	1	46.91

Table 2: Proteins regulated in Schizophrenic brains, classified according to their biological function. Proteins marked with (*) are encoded by genes that map to genomic regions previously associated with Schizophrenia. The standard deviation for all H/L ratios is +/- 0,00 and the accession numbers are from the Swiss-Prot database. (Abbreviations: pI (th) – predicted protein isoeletric point; MW (th) - predicted protein molecular weight; Chr Loci – Gene *Locus;* Id. Pept – Number of peptides that identified the protein; MASCOT Score – identified peptide database search score).

Functional class	***Total altered proteins***	***Schizophrenia***	
		Upregulated	**Downregulated**
Transport	3	2	1
Cell communication Signal transduction	11	3	8
Energy metabolism	9	1	8
Cell growth/maintenance	10	1	9
Protein metabolism	6	0	6
Nucleic acid metabolism	2	0	2
Immune response *Neurogenesis* *Synaptic Transmission* *Oligodendrocyte Metabolism*	3	0	3
Unknown	1	0	1
Total	45	7	38

Table 3: Regulated proteins in SCZ ATL according to their functional classification.

Table 4: Proteins found regulated in ATL SCZ that have previously been described in other reports as relevant in SCZ. Genes/proteins marked with an asterisk were indirectly validated by those correspondent authors.

Gene symbol	Product Name	Type of analysis	Tissue	Described as regulated by:
MOG*	Myelin Oligodendrocyte glycoprotein	1- microarray and qPCR 2- microarray 3- microarray and qPCR	1- pre-frontal cortex 2- several brain region 3- pre-frontal cortex	1- Tkachev et al., 2003 2- Katsel et al., 2005 3- Arion et al., 2007
MBP*	Myelin Basic Protein	1- immunoassay 2 - microarray and qPCR	1- anterior frontal cortex 2- pre-frontal cortex	1- Honer et al., 1999 2-Tkachev et al., 2003
GFAP*	Glial fibrillary acidic protein	1- proteomics 2- *in situ* hybridization 3- microarray and qPCR	1- frontal cortex 2- cingulate cortex 3- pre-frontal cortex	1- Johnston-Wilson et al., 2000 2- Webster et al., 2005 3- Tkachev et al., 2003
CNP*	2',3'-cyclic-nucleotide 3'-phosphodiesterase	1- microarray 2- microarray and qPCR 3- microarray 4- microarray and proteomics 5- microarray 6- microarrays 7- qPCR	1- cortical tissues 2- pre-frontal cortex 3- Temporal Gyrus 4- pre-frontal cortex 5- several brain region 6- anterior cingulate cortex and hippocampus 7 - anterior cingulate córtex	1- Hakak et al., 2001 2- Tkachev et al., 2003 3- Aston et al., 2004 4- Prabakaran et al., 2004 5- Katsel et al., 2005 6- Dracheva et al., 2006. 7- McCullumsmith et al., 2007
CAN*	Calcineurin	1 & 2- microarray	1- cortical tissues 2- hippocampus	1- Hakak et al., 2001 2- Eastwood et al., 2005
SPTAN1*	Spectrin alpha chain (fodrin)	Western blot	left superior temporal cortices	Kitamura et al., 1998
14-3-3 eta (YWHAH) *	Protein kinase C inhibitor protein 1 (eta)	1- in situ hybridization 2- microarray	1- pre-frontal cortex 2- cerebellum	1- Middleton et al., 2005 2- Vawter et al., 2001
14-3-3 zeta/delta (YWHAZ) *	Protein kinase C inhibitor protein 1 (zeta/delta)	1- in situ hybridization 2- proteomics	1- pre-frontal cortex 2- corpus callosum	1- Middleton et al., 2005 2- Sivagnanasundaram et al., 2007
14-3-3 gamma (YWHAG) *	Protein kinase C inhibitor protein 1 (gamma)	1- in situ hybridization 2- proteomics	1- pre-frontal cortex 2- corpus callosum	1- Middleton et al., 2005 2- Sivagnanasundaram et al., 2007
ALDOC*	Fructose bisphosphate aldolase C	1- proteomics 2- microarray and	1- anterior cingulate cortex	1- Clark et al., 2006 2- Prabakaran et al.,

Gene symbol	Product Name	Type of analysis	Tissue	Described as regulated by:
		proteomics 3- proteomics 4- immunoassay*	2- pre-frontal cortex 3- frontal cortex 4- human CSF	2004 3- Johnston-Wilson et al., 2000 4- Willson et al., 1980
HIST1H3A*	histone H3.1	immunoblotting, immunohistochemical, microarray and qPCR	pré-frontal córtex	Akbarian et al., 2005
CA2	Carbonic Anhydrase 2	1- proteomics 2- proteomics	1- frontal cortex 2- anterior cingulate cortex	1- Johnston-Wilson et al., 2000 2- Beasley et al., 2006
RPS10	40S ribosomal protein – family S10	microarray	pré-frontal córtex	Vawter et al., 2002
UBE2N	Ubiquitin	microarray	pré-frontal córtex	Vawter et al., 2002
EBP50	Ezrin-radixin-moesin-binding phosphoprotein 50	microarray	peripheral blood lymphocytes	Bowden et al., 2006
TUBB2B	Tubulin beta polypeptide	1- in situ hybridization 2- proteomics	1- frontal cortex 2- corpus callosum	1- Virgo et al., 1995 2- Sivagnanasundaram et al., 2007
NEFL	Neurofilament triplet L protein	proteomics	corpus callosum	Sivagnanasundaram et al., 2007
NEFM	Neurofilament triplet M protein	proteomics	corpus callosum	Sivagnanasundaram et al., 2007

Capítulo 6:

Análise do Proteoma de Córtex Pré-Frontal Cerebral Humano Utilizando **Shotgun Proteomics**.

Através de um manuscrito submetido ao periódico "Journal of Neurology" descrevemos os dados por nós revelados do proteoma de córtex pré-frontal humano por *Shotgun Proteomics*.

Rapid communication

Proteome analysis of human dorsolateral prefrontal cortex using shotgun mass spectrometry

Daniel Martins de Souza[1,2,3], Wagner F. Gattaz[1], Andrea Schmitt[4,5], Christiane Rewerts[2], Sérgio Marangoni[3], José C. Novello[3], Giuseppina Maccarrone[2], Emmanuel Dias-Neto[1,6] & Christoph W. Turck[2].

1- Laboratório de Neurociências – Instituto de Psiquiatria – Faculdade de Medicina – Universidade de São Paulo - Rua Dr. Ovídio Pires de Campos, no 785, São Paulo, SP, CEP 05403-010, Brazil.
2- Max Planck Institute of Psychiatry - Kraepelinstrasse 2, D-80804 – Munich - Germany.
3- Laboratório de Proteômica – Departamento de Bioquímica – Instituto de Biologia, CP 6109, Universidade Estadual de Campinas – UNICAMP, Campinas, SP, CEP 13083-970, Brazil.
4- Department of Psychiatry, Von Siebold Str. 5, University of Goettingen, 37075 Goettingen, Germany.
5- Central Institute of Mental Health, J5, 68159 Mannheim, Germany.
6- Present address: University of Texas, MD Anderson Cancer Center, 1515 Holcombe Blvd, 77030, Houston, Texas, USA.

Corresponding Author:
Daniel Martins
Laboratório de Proteômica - Depto de Bioquímica, Instituto de Biologia.
UNICAMP, Campinas, SP, Brazil - CEP 13083-970.
java99@unicamp.br / http://www.proteome.ibi.unicamp.br

Keywords: proteome, prefrontal cortex, shotgun proteomics, mass spectrometry, brain

Abstract:

A shotgun proteome analysis that included isoelectric focusing fractionation, reversed phase liquid chromatography, and MALDI-TOF/TOF mass spectrometric analysis of tryptic digests from a pool of seven human dorsolateral prefrontal cortex protein extracts was performed and 387 expressed proteins from these samples involved in different biochemical pathways were identified by two or more peptides with high confidence search scores. This analysis contributes to the knowledge of the human brain proteome and with future applications in basic and clinical researches.

Dorsolateral prefrontal cortex (DLPFC) is the anterior region of the frontal lobes situated above the motor and premotor areas. It is the neocortical region most elaborated in primates to provide a diverse and flexible behavioral repertoire. The DLPFC is responsible for the execution of high functions such as differentiation of conflicting thoughts, determination of concepts of right and wrong, adequate social behavior and personality expression [1,2]. Neurocircuitry defects in DLPFC have been related to the biology of several neuropsychiatric conditions such as Tourette's syndrome, Huntington's disease, obsessive-compulsive disorder, attention-deficit/hyperactivity disorder, schizophrenia, and mood disorders [3]. Besides, DLPFC dysfunction has been described in bipolar disorder [4], Parkinson disease [5], major depression [6] and Alzheimer disease [7].

The proteome within brain regions changes qualitatively and quantitatively during aging and in different disease states. Thus the identification of the proteomes from discrete brain regions, related to pathological or non-pathological conditions may contribute to a better functional understanding of those areas, as well as to the identification of new and specific therapeutic targets. In the present work, we performed a shotgun proteome analysis of a protein pool from dorsolateral prefrontal cortex (DLPFC - Brodmann area 46) of seven individuals free from neuropsychiatric diseases. The shotgun mass spectrometry method used here is superior in this effort compared to other techniques such as two-dimensional gel electrophoresis (2-DE) followed by a mass spectrometry (MS) approach [8-11].

Post-mortem brain samples from the DLPFC tissue (BA46) were collected from seven individuals (Table 1) from the *Central Institute of Mental Health* (Mannheim, Germany). On average, the samples were collected 27.1 hours after death. Patients were classified, *ante mortem,* as controls by an experienced psychiatrist according to the criteria of the DSM IV [12]. All assessments and *post mortem* evaluations and procedures were previously approved by the Ethics Committee of the Faculty of Medicine of Heidelberg University, Germany. Fifty milligrams of the human DLPFC from seven control subjects were individually ground with glass spheres in 200µl of 6M guanidine and 0.1M HEPES buffer. Total protein from the DLPFC pool (100 µg) was reduced using DTT and alkylated with 1 ml of 0.4 M iodoacetamide. Protein samples were digested in 200 mM NH_4HCO_3, pH 8.3 with 1mg/ml trypsin at a ratio of 1:50 (protein:enzyme) at 37° C for 4h. Resulting peptides were fractionated on immobilized pH gradient strips (IPG 17cm), pH 3.5-4.5. The

strips were rehydratated for 12h and run for 8h with a constant voltage of 10,000V. Each strip was manually cut in 47 pieces and the peptides extracted with 1% formic acid. Each of the 47 peptide samples from isoelectric focusing was further fractionated on a micro-LC-System (HP1100 Agilent Technologies, Waldbronn, Germany) using an RP-C-18 monolithic column (200µm id. x 5cm, Dionex, Sunnyvale, CA) with a flow rate of 4 µl/min and a 40 min gradient from 10 to 100 % of solvent B (acetonitrile; 0.1% trifluoroacetic acid). Each isoelectric focusing eluate was chromatographed and fractions were collected onto Prespotted AnchorChip targets (Bruker Daltonics, Bremen, Germany) and providing matrix thin layer preparations using a PROTEINEER-FC robot (Bruker Daltonics). Mass spectra from each target spot were acquired fully automatically using an Ultraflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics). WARP-LC 1.0 software was used for spectra acquisition and controlling the automatic selection of peptides for further MS/MS analysis. Acquired MS and MS/MS spectra were automatically sent as combined peak lists by the WARP-LC 1.0 to Biotools software 3.0 (Bruker Daltonics) and searched against the NCBI database (Dec.16th, 2006) using an in-house version of MASCOT 2.1 (Matrix Science, London, UK).

The shotgun analysis of DLPFC revealed 1,161 non-redundant proteins that were assigned with 95% confidence, including 387 (33.3%) proteins identified with two or more peptides. Since the recognition of proteins identified by a single peptide should be considered provisional, further analyses were undertaken with the set of 387 non-redundant proteins identified with two or more peptides. Most of these proteins, 226 (58.4%), were identified by 2 peptides, while 41 (10.6%) were identified by 3 peptides and another 120 (31%) proteins were identified by 4 or more peptides (Figure 1). These proteins are shown in the supporting information that accompanies this article.

The 387 proteins identified by more than 1 peptide show a diverse range of biophysical characteristics, with isoeletric points (pI) rainging from 4.09 to 11.55 and molecular weights (MW) varying from 2.9 kDa to 532.4 kDa. Approximately half of the identified proteins have a MW below 50 kDa (Figure 2A) and roughly one third have pIs >7 (Figure 2B). This demonstrates the superior performance of the shotgun mass spectrometry approach over 2-DE, where proteins with these biophysical characteristics are

difficult to identify [13]. In addition, this method significantly improves the identification of low abundant proteins [8,14,15].

The identified proteins were then classified according to their putative biological process with the help of the Human Proteome Reference Database (HPRD – http://www.hprd.org). Biological processes related to 22.5% of the proteins identified here have not been defined yet (Figure 3). Pan et al. [16] revealed that 28.2% of the frontal cortex proteome, determined with a shotgun method, could not be assigned to biological processes while a much lower fraction (3.2%) had the same behavior, when the temporal lobe proteome was evaluated with 2-DE followed by mass spectrometry (MS) to protein identification [17]. This reinforces the notion that shotgun proteomics is capable and more efficient in the identification of less-abundant, and thus, usually less-characterized proteins, that can be important players in neurological diseases and relevant targets to pharmaceutical applications.

The most abundantly populated functional classes include cell growth and/or maintenance (17.6%), metabolism/energy pathways (16.5%), and cell communication/signal transduction (13.7%). Other processes also identified are protein metabolism, transport, regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism and immune response (Figure 3).

These proteins were also classified according to their sub-cellular localization using the “Proteome Analyst Specialized Subcellular Localization Server“ software [18]. Putative subcellular localizations could be defined for 65% of the proteins. As the sample preparation method used here favors the analysis of proteins with cytoplasmic localization, it is not a surprise to see that most of the identified proteins (55.4%) are indeed predicted to be located in the cytoplasm. Besides, 9.2% of evaluated proteins are membrane proteins, including many cellular receptors that are major players in a number of neurophysiologic processes and are potential therapeutic targets. Others include nuclear proteins (15.3%) and extracellular proteins that are important in gene expression control and cellular communication, respectively (Figure 4).

The 10 most abundant proteins we found were Glial fibrillary acidic protein (GFAP), Myelin basic protein (MBP), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Spectrin alpha (SPTAN1), Tubulin alpha-1A chain (TUBA1A), Plectin-1

(PLEC1), Beta-spectrin 2 (SPTBN1), Histone H3/b (HIST1H3B) and Alpha enolase (ENO1) (the entire set of identified proteins is presented in the Supporting Information). These 10 proteins are known as important players in brain metabolism, and are involved in important structures such as cytoskeleton and myelin sheath, as well as in the mitochondrial metabolism. Of note is the fact that the top 10 proteins have a wide range of pIs and MWs, including 4 proteins more basic than 7 and 3 proteins bigger than 200 kDa. Proteins with these physicochemical characteristics are usually less represented when 2-DE is used and demonstrate the strength of the shotgun mass spectrometry approach for a better definition of the human neuroproteome.

Other brain proteome studies using 2-DE have been mainly used to reveal differentially expressed proteins, and lists of proteins categorized by their abundance are lacking from the scientific literature. Examples of differential protein analysis include the comparison of prefrontal cortex (PFC) that revealed 18 differentially expressed proteins in human alcoholics [19] and the identification of 5 differentially expressed proteins in PFC of suicide victims [20]. Moreover, Prabakaran et al. [21] presented 50 differentially expressed proteins in schizophrenic PFC, using a fluorescent 2-DE approach.

Brain proteome studies using shotgun proteomics can reveal a significant fraction of the proteome, in addition to differentially expressed proteins. Combining sample fractionation, 1-D SDS-PAGE, and MS analysis, Park et al. [22] were able to identify more than 1,500 proteins in temporal lobe tissue from epileptic patients. In another shotgun study with human temporal lobe tissue using LC-ESI-MS/MS and gas-phase fractionation, 209 proteins were identified [23].

We believe that the identification of proteins expressed in the DLPFC brain specimens, as well as in other brain areas, will help in gaining a better understanding of brain biochemistry. Moreover, the collection of brain proteome data can be used as a reference for future studies, and a better knowledge of the neuroproteome in health and in illness will certainly impact the diagnosis of many diseases. In the future, it may also help to elaborate more comprehensive disease classifications, when we would be able to determine if conditions that share similar symptoms also have the same underlying molecular basis.

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The authors have none conflict of interest.

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Figure 1: Number of peptides per identified protein in DLPFC.

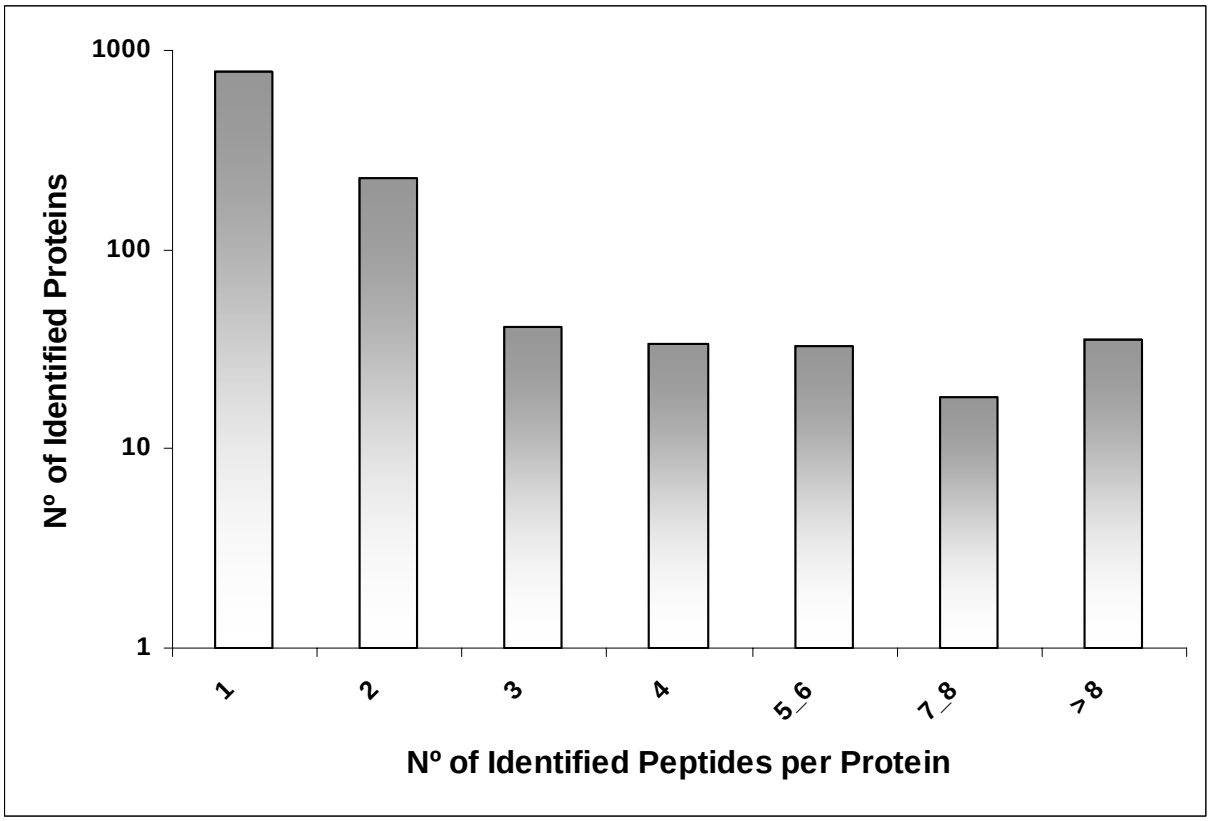
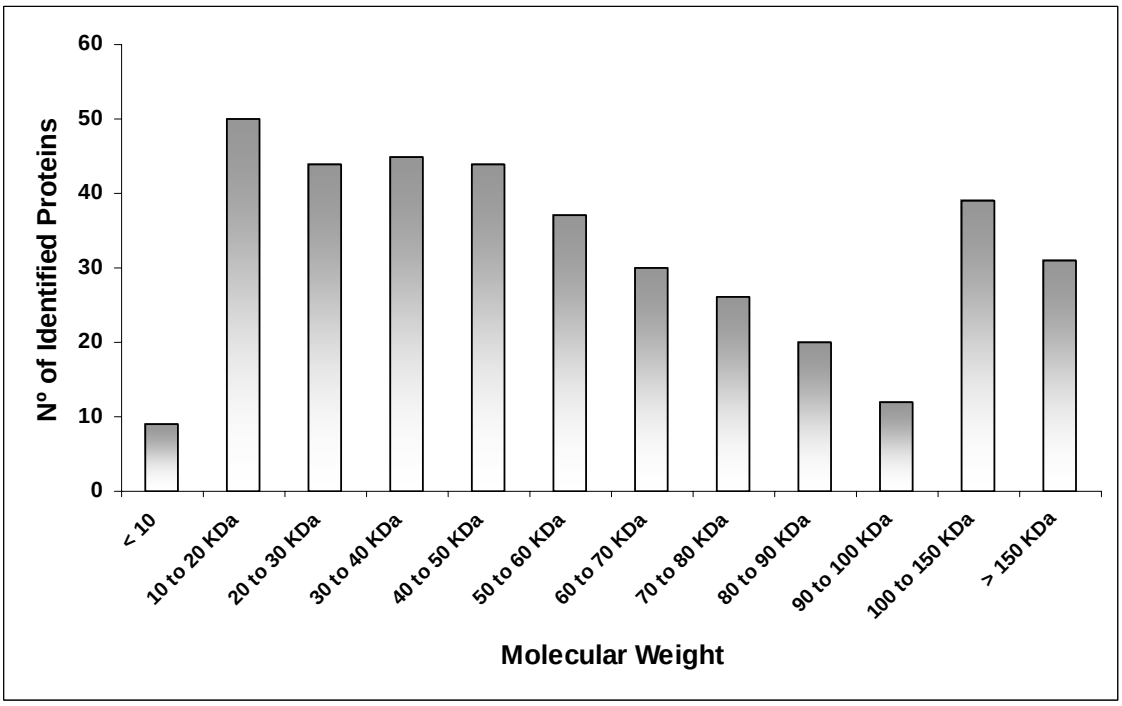


Figure 2: **A**: Molecular weight distribution of DLPFC proteins identified with two or more peptides. **B:** Isoelectric point distribution of DLPFC proteins identified with two or more peptides.



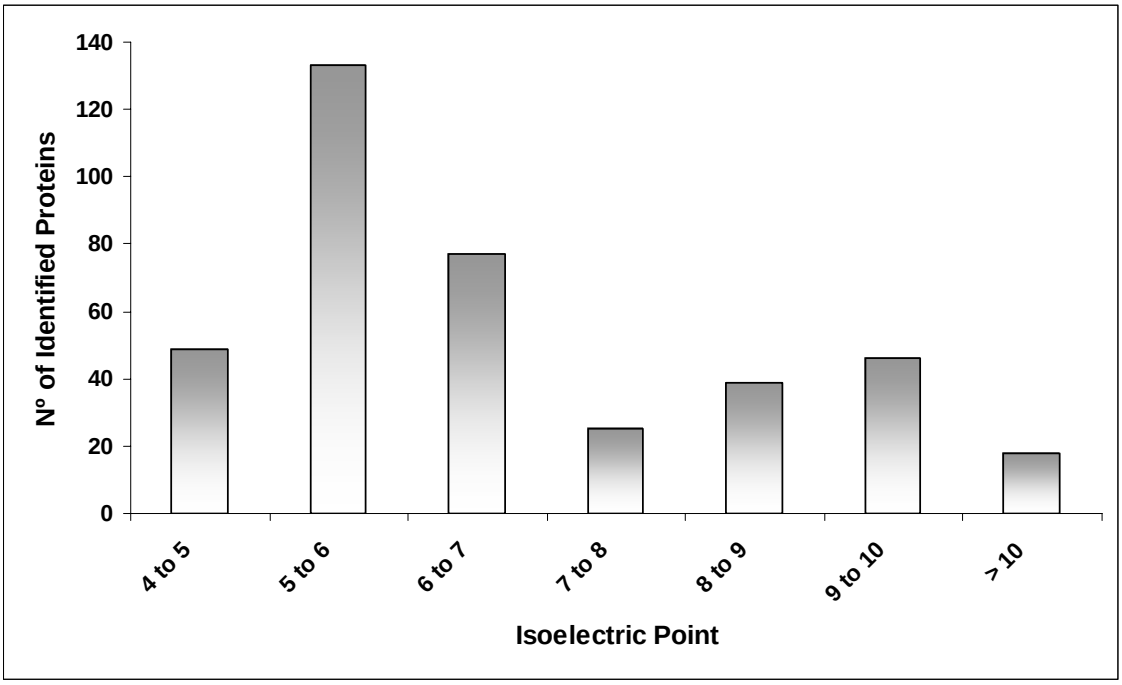


Figure 3: Human Proteome Reference Database analysis of DLPFC proteins identified with two or more peptides.

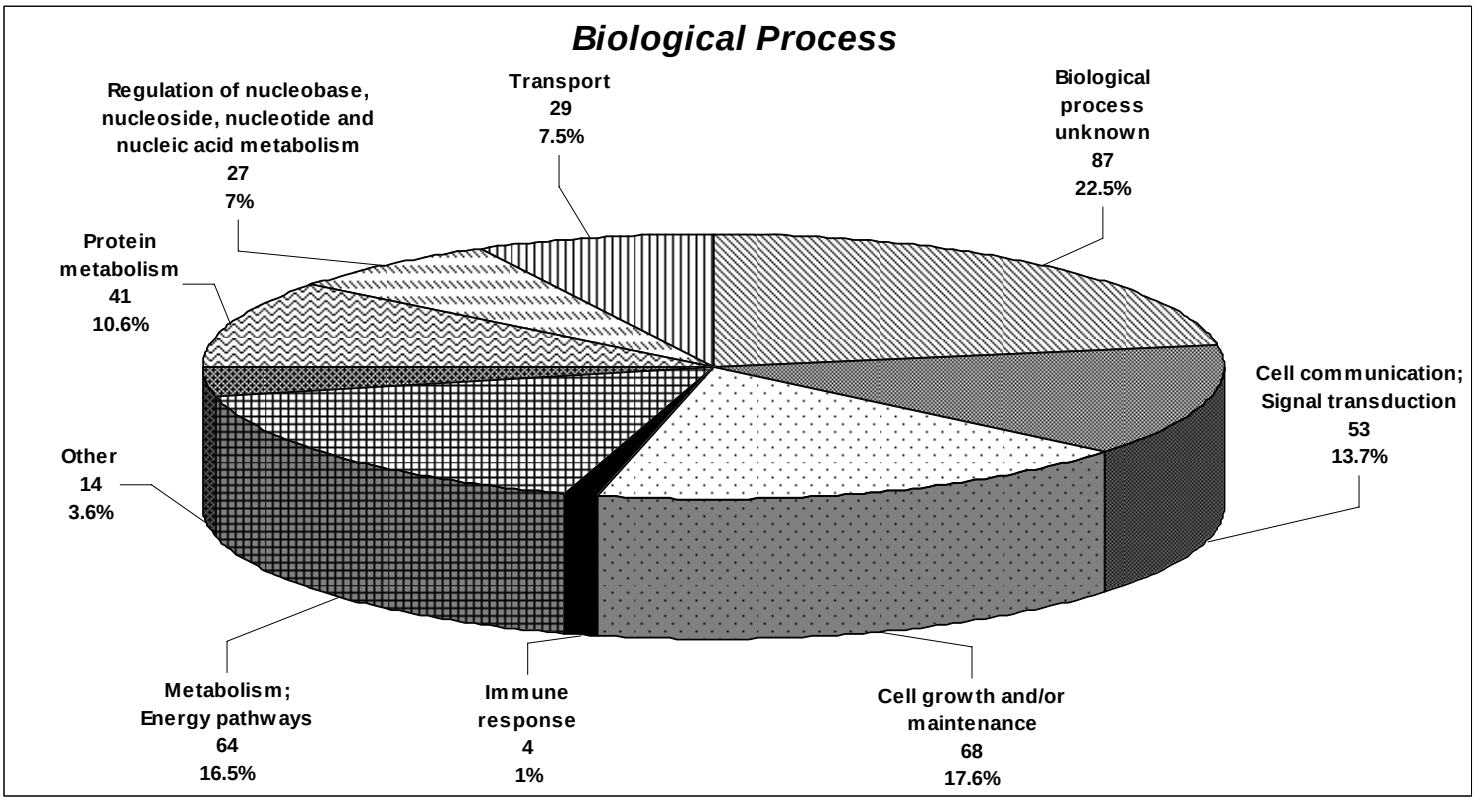
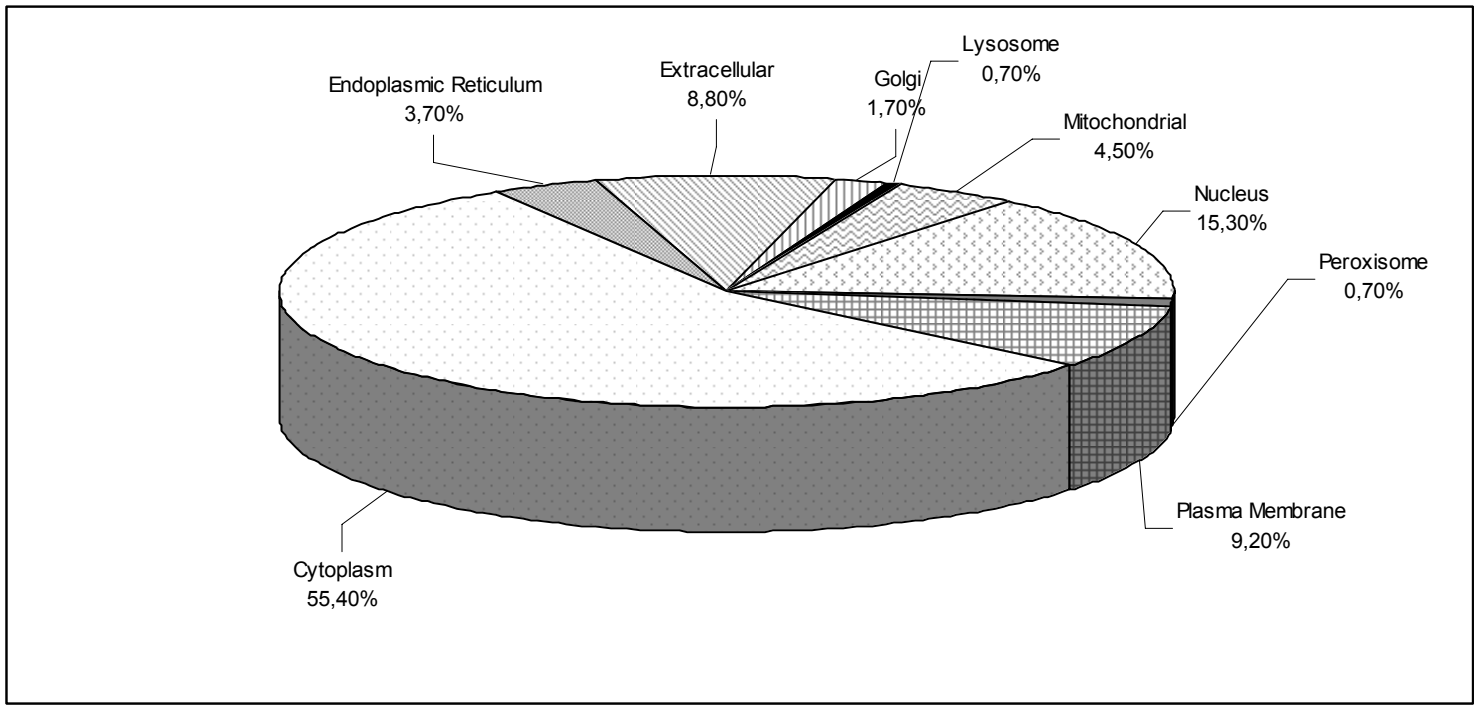


Figure 4: Sub-cellular localization analysis of DLPFC proteins identified with two or more peptides.



Sample ID	Age (years)	Gender	PMI (hours)	pH	Cause of Death	Cigarettes	Alcohol
02/02	41	M	7	7.1	heart infarction	No	No
43/01	91	F	16	6.8	cardio-pulmonary insuffiency	No	No
50/02	69	F	96	6.4	lung emboly	No	No
51/02	57	M	24	6.5	heart infarction	No	No
57/02	53	M	18	7.1	heart infarction	No	No
59/02	63	M	13	6.9	heart infarction	No	No
61/01	66	M	16	6.5	heart infarction	No	No

Table 1: Clinical data of DLPFC samples.

Supporting Information: List of proteins identified in DLPFC with pI, MW and biological process data organized according proteins abundances.

Access name	Protein name	Peptides per protein	pI (th)	MW (th)	Biological Process
GFAP	Glial fibrillary acidic protein	64	5.40	49880	Cell growth and/or maintenance
MBP	Myelin basic protein	32	9.79	33117	Immune response ; Neurogenesis
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	29	8.57	36053	Metabolism ; Energy pathways
SPTAN1	Spectrin alpha chain, brain (Fodrin alpha)	28	5.22	284539	Cell growth and/or maintenance
TUBA1A	Tubulin alpha-1A chain	28	4.94	50136	Cell growth and/or maintenance
PLEC1	Plectin-1	27	5.73	531733	Cytoskeletal anchoring
SPTBN1	Beta-spectrin 2 isoform 2.- Homo sapiens (Human).	24	5.39	274609	Cell growth and/or maintenance
HIST1H3B	Histone H3/b	24	11.13	15404	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
ENO1	Alpha enolase	23	7.01	47169	Metabolism ; Energy pathways
CKB	Creatine kinase B-type	22	5.34	42644	Metabolism ; Energy pathways
TPPP	Tubulin polymerization-promoting protein	21	9.48	23694	Cell growth and/or maintenance
STXBP1	Syntaxin-binding protein 1	20	6.50	67569	Neurotransmitter transport
NEFM	Neurofilament triplet M protein (160 kDa neurofilament protein)	19	4.90	102448	Cell growth and/or maintenance
PEBP1	Phosphatidylethanolamine-binding protein 1	19	7.01	21057	Cell communication ; Signal transduction
TUBB2B	Tubulin beta-2B chain	19	4.78	49953	Cell growth and/or maintenance
CNP	2',3'-cyclic-nucleotide 3'-phosphodiesterase	18	9.17	47579	Metabolism ; Energy pathways
HSPA2	Heat shock-related 70 kDa protein 2	18	5.56	70021	Protein metabolism
NEFL	Neurofilament triplet L protein (68 kDa neurofilament protein)	18	4.64	61517	Cell growth and/or maintenance
ALDOC	fructose-bisphosphate aldolase C - human	17	6.41	39456	Metabolism ; Energy pathways
CLTC	Clathrin heavy chain 1	16	5.48	191615	Cell growth and/or maintenance
DPYSL3	Dihydropyrimidinase-like 3	15	6.04	61979	Metabolism ; Energy pathways
ATP5A1	ATP synthase subunit alpha, mitochondrial	14	9.16	59751	Metabolism ; Energy pathways
ATP5B	ATP synthase subunit beta, mitochondrial [Precursor]	13	5.26	56560	Metabolism ; Energy pathways
HBB	Hemoglobin subunit beta	13	6.74	15998	Transport
MAP1A	Microtubule-associated protein 1A	13	4.86	306482	Cell growth and/or maintenance
TUBB4	Tubulin beta-4 chain	13	5.55	88382	Cell growth and/or maintenance
GPX1	Glutathione peroxidase 1	12	6.15	21899	Anti-apoptosis
ACTG1	actin gamma	11	5.31	41792	Cell growth and/or maintenance
GOT1	Aspartate aminotransferase, cytoplasmic	11	6.53	46248	Metabolism ; Energy pathways
VAMP2	Vesicle-associated membrane protein 2 (Synaptobrevin 2)	11	7.84	12649	Transport
DNM1	Dynamin 1.- Homo sapiens (Human).	10	6.73	97408	Cell communication ; Signal transduction
RP11-631M21.2	Novel protein similar to beta-tubulin 4Q [Fragment]	10	4.95	48726	Biological_process unknown
UBE2D4	Ubiquitin-conjugating enzyme E2 D4	10	6.88	16649	Protein metabolism
MAP1B	Microtubule-associated protein 1B	9	4.73	270620	Cell growth and/or maintenance
DKFZp686L04275	Putative uncharacterized protein DKFZp686L04275 [Fragment]	9	9.21	53969	Biological_process unknown
ATP4B	ATPase, H+/K+ exchanging, beta polypeptide	8	6.98	33367	Metabolism ; Energy pathways
CALM1	calmodulin	8	4.09	16838	Cell communication ; Signal transduction
MAPT	Microtubule-associated protein tau	8	6.25	78878	Cell growth and/or maintenance
NEFH	Neurofilament heavy polypeptide	8	5.89	112480	Cell growth and/or maintenance
PRDX1	Peroxiredoxin-1	8	8.27	22110	Metabolism ; Energy pathways
PACSIN1	Protein kinase C and casein kinase substrate in neurons 1	8	5.26	46146	Cell communication ; Signal transduction
SYN1	Synapsin-1	8	9.84	74111	Transport
ANXA6	Annexin A6	7	5.42	75873	Cell communication ; Signal transduction
EPB41L3	Band 4.1-like protein 3	7	5.09	120678	Cell growth and/or maintenance
CHCHD3	Coiled-coil-helix-coiled-coil-helix domain-containing protein 3	7	8.48	26152	Biological_process unknown

HSPA8	HSPA8 protein [Fragment]	7	5.36	64673	Protein metabolism
KIF21A	Kinesin-like protein KIF21A	7	6.05	187179	Cell growth and/or maintenance
MAP2	Microtubule-associated protein 2	7	4.83	199539	Cell growth and/or maintenance
NCAM1	Neural cell adhesion molecule 1	7	4.77	83770	Cell communication ; Signal transduction
NFASC	Neurofascin [Precursor]	7	6.24	150027	Cell communication ; Signal transduction
Q9UCR6_HUMAN	P60, 60-kDa heat shock protein, HSP60 [Fragment]	7	4.58	2991	Biological_process unknown
PKM2	Pyruvate kinase isozymes M1/M2	7	7.96	57937	Metabolism ; Energy pathways
TUBA1B	TUBA1B protein	7	4.87	37218	Cell growth and/or maintenance
CRYAB	Alpha-crystallin B chain	6	6.76	20159	Protein metabolism
GPI	Glucose-6-phosphate isomerase	6	8.42	63147	Metabolism ; Energy pathways
HK1	Hexokinase-1	6	6.36	102486	Metabolism ; Energy pathways
LMNB2	Lamin-B2	6	5.29	67689	Cell growth and/or maintenance
PPWD1	Peptidylprolyl isomerase domain and WD repeat-containing protein 1	6	6.70	73575	Biological_process unknown
PCBD2	Pterin-4-alpha-carbinolamine dehydratase 2	6	6.97	11761	Metabolism ; Energy pathways
PDHA1	Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial	6	8.35	43296	Metabolism ; Energy pathways
PPP3CA	Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform	6	5.58	58688	Cell communication ; Signal transduction
VIM	Vimentin	6	5.06	53652	Cell growth and/or maintenance
AMPH	Amphiphysin	5	4.58	76257	Cell communication ; Signal transduction
ATP2B2	Ca2+-transporting ATPase (EC 3.6.3.8) 2, long splice form - human	5	5.66	136876	Transport
Q7LDD5_HUMAN	Calmodulin-dependent protein kinase II alpha	5	6.61	54088	Cell communication ; Signal transduction
EFHD2	EF-hand domain-containing protein 2	5	5.15	26697	Biological_process unknown
ENO2	Gamma-enolase	5	4.91	47269	Metabolism ; Energy pathways
GNAO1	Guanine nucleotide-binding protein G(o) subunit alpha 1	5	5.34	40051	Cell communication ; Signal transduction
HSPA8	Heat shock cognate 71 kDa protein	5	5.37	70898	Protein metabolism
HSP90AB1	Heat shock protein 90kDa alpha (Cytosolic), class B member 1 [Fragment]	5	4.98	19135	Cell communication ; Signal transduction
HNRPU	Heterogeneous nuclear ribonucleoprotein U [Fragment]	5	8.06	79844	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
HIST1H4A	Histone H4	5	11.36	11367	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
IDH2	Isocitrate dehydrogenase [NADP], mitochondrial [Precursor]	5	8.88	50909	Metabolism ; Energy pathways
PGK1	Phosphoglycerate kinase 1	5	8.30	44615	Metabolism ; Energy pathways
PSMB4	Proteasome subunit beta type	5	5.70	29190	Protein metabolism
PARK7	Protein DJ-1	5	6.33	19891	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
Q96BE0_HUMAN	Putative uncharacterized protein [Fragment]	5	4.80	29361	Biological_process unknown
DKFZp564P0562	Putative uncharacterized protein DKFZp564P0562 [Fragment]	5	5.37	152088	Biological_process unknown
DKFZp686B23205	Putative uncharacterized protein DKFZp686B23205 [Fragment]	5	5.12	33009	Biological_process unknown
ARHGDIA	Rho GDP-dissociation inhibitor 1	5	5.03	23207	Cell growth and/or maintenance
RTN4	RNT4	5	4.52	108450	Biological_process unknown
SFXN1	Sideroflexin-1	5	9.22	35619	Transport
SIRPB1	Signal regulatory protein beta-1 [Precursor]	5	6.06	43255	Cell communication ; Signal transduction
CCT8	T-complex protein 1 subunit theta	5	5.42	59621	Protein metabolism
TPM4	Tropomyosin alpha-4 chain	5	4.67	28522	Cell growth and/or maintenance
TUBA4A	Tubulin alpha-4A chain	5	4.95	49924	Cell growth and/or maintenance
INA	Alpha-internexin	4	5.34	55391	Cell growth and/or maintenance
ANKMY2	Ankyrin repeat and MYND domain-containing protein 2	4	5.88	49299	Biological_process unknown
ATP1A1	ATPase, Na+/K+ transporting, alpha 1 polypeptide [Fragment]	4	8.48	18172	Transport
CAMK2G	Calcium/calmodulin-dependent protein kinase type II gamma chain	4	7.90	62609	Cell communication ; Signal transduction
CANX	Calnexin [Precursor]	4	4.47	67568	Protein folding
Q7L9H8_HUMAN	similar to HYPOTHETICAL GTP-BINDING PROTEIN IN SEH1-PRP20	4	5.58	73142	Biological_process unknown
CUL3	Cullin-3	4	8.68	88930	Protein metabolism
UQCRB	Cytochrome b-c1 complex subunit 7	4	8.73	13530	Metabolism ; Energy pathways

OPA1	Dynamin-like 120 kDa protein, mitochondrial [Precursor]	4	7.87	111658	Cell communication ; Signal transduction
GDI2	GDP dissociation inhibitor 2 [Fragment]	4	8.98	17783	Transport
GLULD1	Glutamate-ammonia ligase (Glutamine synthetase) domain containing 1	4	5.96	57278	Metabolism ; Energy pathways
GSTP1	Glutathione S-transferase P	4	5.43	23356	Metabolism ; Energy pathways
HSPH1	Heat shock 105kDa/110kDa protein 1	4	5.29	92255	Protein metabolism
Q9BWA9_HUMAN	HNRPA2B1 protein	4	4.79	28412	Biological_process unknown
LETM1	Leucine zipper-EF-hand-containing transmembrane protein 1, mitochondrial [Precursor]	4	6.30	83354	Cell communication ; Signal transduction
MAP1LC3B	Microtubule-associated proteins 1A/1B light chain 3B [Precursor]	4	8.89	14688	Biological_process unknown
MFN2	Mitofusin-2	4	6.52	86402	Metabolism ; Energy pathways
DDAH1	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	4	5.53	31122	Metabolism ; Energy pathways
NDUFS5	NADH dehydrogenase [ubiquinone] iron-sulfur protein 5	4	9.27	12518	Metabolism ; Energy pathways
OXCT	OXCT protein	4	7.13	56192	Biological_process unknown
PSAT1	Phosphoserine aminotransferase	4	7.56	40423	Metabolism ; Energy pathways
PHB	Prohibitin	4	5.57	29832	Cell communication ; Signal transduction
PDIA3	Protein disulfide-isomerase A3 [Precursor]	4	5.98	56782	Protein Metabolism
DKFZp547P162	Putative uncharacterized protein DKFZp547P162 [Fragment]	4	4.86	36247	Biological_process unknown
RPH3A	Rabphilin-3A	4	8.62	76872	Transport
RPL26	RPL26 protein	4	10.55	17244	Protein metabolism
ATP2A2	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	4	5.23	114757	Transport
Q7KYV2_HUMAN	Septin [Fragment]	4	6.10	44158	Cell cycle
ALB	Serum albumin	4	5.92	69367	Transport
ATP1A3	Sodium/potassium-transporting ATPase subunit alpha-3	4	5.22	111749	Transport
SPTBN2	Spectrin beta chain, brain 2	4	5.79	271295	Cell growth and/or maintenance
TPM3	Tropomyosin 3	4	4.81	26282	Cell growth and/or maintenance
CAH05698	unnamed protein product	4	4.84	21801	Biological_process unknown
NSF	Vesicle-fusing ATPase	4	6.52	82560	Metabolism ; Energy pathways
ACTN1	alpha-actinin 1 - human	3	5.25	103057	Cell growth and/or maintenance
ADD1	Alpha-adducin	3	5.60	80955	Cell growth and/or maintenance
ANK2	Ankyrin-2	3	5.03	430344	Cell growth and/or maintenance
MTHFD2	Bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase, mitochondrial	3	8.86	37895	Metabolism ; Energy pathways
CNTNAP1	Contactin-associated protein 1 [Precursor]	3	6.61	156267	Cell communication ; Signal transduction
CRMP1	CRMP1 protein [Fragment]	3	8.94	43746	Cell growth and/or maintenance
DLG4	Disks large homolog 4	3	5.58	80495	Cell communication ; Signal transduction
O62493	DJ366N23.2 (Putative C. elegans UNC-93 (Protein 1, C46F11.1)	3	9.59	27457	Biological_process unknown
EIF4A1	Eukaryotic initiation factor 4A-I	3	5.32	46154	Protein metabolism
SLC9A3R1	Ezrin-radixin-moesin-binding phosphoprotein 50	3	5.55	38868	Cell communication ; Signal transduction
GLUD1	glutamate dehydrogenase 1 precursor	3	7.66	61398	Metabolism ; Energy pathways
MAP6	GTPase, IMAP family member 6 [Fragment]	3	9.34	77071	Cell growth and/or maintenance
HEBP1	Heme-binding protein 1	3	5.71	21097	Biological_process unknown
HNRPK	Heterogeneous nuclear ribonucleoprotein K	3	5.46	47557	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
HSPA9	HSPA9 protein [Fragment]	3	6.04	73854	Protein metabolism
HAPLN2	Hyaluronan and proteoglycan link protein 2 precursor	3	9.12	37775	Cell growth and/or maintenance
LMNA	Lamin-A/C	3	6.57	79397	Cell growth and/or maintenance
NAALADL1	N-acetylated-alpha-linked acidic dipeptidase-like protein	3	5.28	80622	Biological_process unknown
NDUFS1	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	3	5.89	79468	Metabolism ; Energy pathways
NRGN	Neurogranin	3	7.72	7618	Cell communication ; Signal transduction
PALM	Paralemmin	3	4.94	42076	Biological_process unknown
FKBP10	Peptidyl-prolyl cis-trans isomerase	3	5.36	64245	Cell communication ; Signal transduction
PHF3	PHD finger protein 3	3	6.52	229481	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism

PGAM1	Phosphoglycerate Mutase 1	3	6.67	28804	Metabolism ; Energy pathways
PRKCG	Protein kinase C gamma type	3	7.27	78448	Cell communication ; Signal transduction
JE0193	Putative uncharacterized protein	3	5.32	12618	Biological_process unknown
DKFZp686A1195	Putative uncharacterized protein DKFZp686A1195	3	6.33	189618	Biological_process unknown
WBSCR1	Putative uncharacterized protein WBSCR1 [Fragment]	3	5.19	14960	Biological_process unknown
TPM1	Sarcomeric tropomyosin kappa	3	4.65	32650	Cell growth and/or maintenance
SVIP	Small VCP/p97-interacting protein	3	9.06	8443	Biological_process unknown
STIP1	Stress-induced-phosphoprotein 1	3	6.40	62639	Cell communication ; Signal transduction
SYNJ1	Synaptojanin-1	3	7.13	173346	Metabolism
VCP	Transitional endoplasmic reticulum ATPase	3	5.14	89322	Metabolism ; Energy pathways
TKT	Transketolase	3	7.58	67878	Metabolism ; Energy pathways
TUBA1C	Tubulin alpha-1C chain	3	4.96	49895	Cell growth and/or maintenance
CAD61726	unnamed protein product	3	9.99	11466	Biological_process unknown
CAE11694	unnamed protein product	3	5.90	35777	Biological_process unknown
CAE99312	unnamed protein product	3	8.62	12182	Biological_process unknown
CAF00100	unnamed protein product	3	8.79	12115	Biological_process unknown
CAF06470	unnamed protein product	3	11.42	39586	Biological_process unknown
ATP6V1A	Vacuolar ATP synthase catalytic subunit A	3	5.35	68304	Metabolism ; Energy pathways
RPS13	40S ribosomal protein S13	2	10.53	17222	Protein metabolism
RPS18	40S ribosomal protein S18	2	10.99	17719	Protein metabolism
RPS7	40S ribosomal protein S7	2	10.09	22127	Protein metabolism
RPL27	60S ribosomal protein L27	2	10.56	15798	Protein metabolism
DBI	Acyl-CoA-binding protein	2	6.12	10044	Metabolism ; Energy pathways
ALDH7A1	Alpha-aminoadipic semialdehyde dehydrogenase	2	6.44	55366	Metabolism ; Energy pathways
AMPD2	AMP deaminase 2	2	6.46	100688	Metabolism ; Energy pathways
AP1GBP1	AP1 subunit gamma-binding protein 1	2	4.90	140654	Transport
APOE	apolipoprotein E precursor	2	5.65	36154	Transport
PEA15	Astrocytic phosphoprotein PEA-15	2	4.93	15040	Cell communication ; Signal transduction
ATP6V1B1	ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B1	2	5.44	56833	Transport
HSPG2	Basement membrane-specific heparan sulfate proteoglycan core protein [Precursor]	2	6.06	468825	Cell growth and/or maintenance
ADD2	Beta-adducin	2	5.67	80854	Cell growth and/or maintenance
DEFB103A	Beta-defensin 3 [Fragment]	2	10.08	5161	Immune response
BYSL	Bystin	2	8.19	49601	Cell growth and/or maintenance
KCNMA1	Calcium-activated potassium channel subunit alpha-1	2	6.66	137560	Transport
CHP	Calcium-binding protein p22	2	4.98	22456	Cell communication ; Signal transduction
CAPN1	Calpain-1 catalytic subunit	2	5.49	81890	Protein metabolism
CSNK2A1	Casein kinase II subunit alpha	2	7.29	45144	Signal transduction ; Protein modification
CTNND2	Catenin delta-2	2	7.78	132656	Cell communication ; Signal transduction
Q6LCG8_HUMAN	Catenin-4 [Fragment]	2	9.40	68009	Cell growth and/or maintenance
CTSB	Cathepsin B	2	5.88	37822	Protein metabolism
ATCAY	Caytaxin	2	4.54	42120	Transport
CDC37	CDC37 protein	2	5.17	44453	Protein metabolism
CDC42EP1	Cdc42 effector protein 1	2	6.64	40295	Cell growth and/or maintenance
Q6ZN96_HUMAN	CDNA FLJ16308 fis, clone PUAEN2006335	2	8.84	63671	Biological_process unknown
Q6ZP37_HUMAN	similar to Galactokinase	2	6.81	42952	Biological_process unknown
Q8NB80_HUMAN	similar to SPLICING FACTOR, ARGININE/SERINE-RICH 7	2	9.96	15862	Biological_process unknown
Q8N9K4_HUMAN	similar to ALPHA-ADAPTIN A	2	5.72	52032	Biological_process unknown
Q6ZTL0_HUMAN	similar to Homo sapiens N-ethylmaleimide-sensitive factor attachment protein, alpha	2	5.02	15808	Biological_process unknown
Q6ZTE7_HUMAN	similar to Homo sapiens ring finger protein 22	2	9.40	62277	Biological_process unknown

Q6ZRA8_HUMAN	similar to Focal adhesion kinase 2	2	5.47	68032	Biological_process unknown
CADM3	Cell adhesion molecule 3 [Precursor]	2	5.71	43300	Immune response
CEP290	Centrosomal protein Cep290	2	5.75	290386	Biological_process unknown
CCT3	Chaperonin containing TCP1, subunit 3	2	6.46	57972	Protein metabolism
CHMP5	Charged multivesicular body protein 5	2	4.68	24571	Transport
SLC44A2	Choline transporter-like protein 2	2	8.93	80152	Cell communication ; Signal transduction
CLTCL1	Clathrin heavy chain 2	2	5.57	187030	Cell growth and/or maintenance
CLIP1	CLIP1 protein [Fragment]	2	7.20	71733	Cell growth and/or maintenance
CLASP1	CLIP-associating protein 1	2	9.14	169451	Cell growth and/or maintenance
Q9H4N1_HUMAN	Clone CDABP0107 mRNA sequence	2	5.22	45665	Biological_process unknown
COPA	Coatomer subunit alpha	2	7.70	138332	Transport
CFL1	Cofilin-1	2	8.22	18502	Cell growth and/or maintenance
CCDC124	Coiled-coil domain-containing protein 124	2	9.54	25835	Biological_process unknown
CHCHD6	Coiled-coil-helix-coiled-coil-helix domain-containing protein 6	2	9.01	26458	Biological_process unknown
CNTN1	Contactin-1	2	5.62	113320	Cell growth and/or maintenance
CORO1A	Coronin-1A	2	6.25	51026	Cell growth and/or maintenance
CORO2B	Coronin-2B	2	8.39	54393	Cell growth and/or maintenance
CTTNBP2	Cortactin-binding protein 2	2	8.23	181051	Biological_process unknown
CSDE1	CSDE1 protein [Fragment]	2	5.31	30108	Regulation of gene expression, epigenetic
CTBP2	C-terminal binding protein 2	2	6.50	56102	Signal transduction ; Cell communication
CAND1	Cullin-associated NEDD8-dissociated protein 1	2	5.52	136376	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
COX6C	Cytochrome c oxidase polypeptide Vic	2	10.38	8781	Metabolism ; Energy pathways
COX5A	Cytochrome c oxidase subunit 5A, mitochondrial	2	6.30	16774	Metabolism ; Energy pathways
COX5B	Cytochrome c oxidase subunit 5B, mitochondrial	2	9.07	13696	Metabolism ; Energy pathways
DENR	Density-regulated protein	2	5.21	22092	Cell communication ; Signal transduction
DES	Desmin	2	5.21	53536	Cell growth and/or maintenance
NUDT10	Diphosphoinositol polyphosphate phosphohydrolase 3 alpha	2	5.52	18500	Metabolism ; Energy pathways
APEX1	DNA-(apurinic or apyrimidinic site) lyase	2	8.33	35555	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
DNAJB1	DnaJ homolog subfamily B member 1	2	8.74	38044	Protein metabolism
DNAJB2	DnaJ homolog subfamily B member 2	2	5.69	35580	Protein metabolism
DNAJC11	DnaJ homolog subfamily C member 11	2	8.54	63278	Biological_process unknown
STT3B	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3B	2	9.04	93674	Biological_process unknown
DCAMKL1	Doublecortin and CaM kinase-like 1	2	5.52	47681	Cell communication ; Signal transduction
DCTN2	Dynactin subunit 2	2	5.10	44231	Cell growth and/or maintenance
DYNC1H1	Dynein heavy chain, cytosolic	2	6.01	532408	Metabolism ; Energy pathways
EEA1	Early endosome antigen 1	2	5.53	162466	Transport
EEF2K	EEF2K protein	2	5.19	82191	Protein metabolism
SLC4A4	Electrogenic sodium bicarbonate cotransporter 1	2	6.35	121461	Transport
ELMOD1	ELMO domain-containing protein 1	2	8.78	39052	Biological_process unknown
ERP29	Endoplasmic reticulum protein ERp29 [Precursor]	2	6.77	28993	Protein folding
HSP90B1	endoplasmin precursor - human	2	4.76	92469	Protein metabolism
KIAA1189	Ermin	2	4.75	32783	Cell motility ; Cytoskeleton organization and biogenesis
EIF2S1	Eukaryotic translation initiation factor 2 subunit 1	2	5.02	36112	Protein metabolism
EIF4B	Eukaryotic translation initiation factor 4B	2	5.55	69166	Protein metabolism
FUBP1	Far upstream element-binding protein 1	2	7.18	67560	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
FASN	Fatty acid synthase	2	5.99	273400	Metabolism ; Energy pathways
FGA	Fibrinogen alpha chain precursor	2	5.70	94973	Protein metabolism
FLJ00128	FLJ00128 protein [Fragment]	2	6.00	167673	Biological_process unknown
FMNL2	Formin-like protein 2	2	6.98	123400	Biological_process unknown

Q86TR5_HUMAN	Full-length cDNA 5-PRIME	2	8.55	8963	Biological_process unknown
FH	Fumarate hydratase, mitochondrial [Precursor]	2	8.85	54637	Metabolism ; Energy pathways
GSPT1	G1 to S phase transition 1	2	5.45	55657	Cell communication ; Signal transduction
GLUD2	GLUD2 protein	2	6.72	29290	Metabolism ; Energy pathways
PYGB	Glycogen phosphorylase, brain form	2	6.40	96696	Metabolism ; Energy pathways
GNAI1	Guanine nucleotide-binding protein G(i), alpha-1 subunit	2	5.69	40361	Cell communication ; Signal transduction
HBS1L	HBS1-like protein	2	6.17	75473	Protein metabolism
HSPA12A	Heat shock 70 kDa protein 12A	2	6.32	74978	Protein folding
HSPA4L	Heat shock 70 kDa protein 4L	2	5.63	94486	Protein metabolism
HSPA1A	Heat shock 70kDa protein 1A	2	5.35	70038	Protein Metabolism
HP1BP3	Heterochromatin protein 1, binding protein 3	2	9.69	61207	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
HNRPA0	Heterogeneous nuclear ribonucleoprotein A0	2	9.34	30841	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
HNRPA1	Heterogeneous nuclear ribonucleoprotein A1	2	9.26	38846	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
HNRPH3	Heterogeneous nuclear ribonucleoprotein H3	2	6.37	36926	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
HIST1H2AE	Histone H2A type 1-E	2	11.05	14135	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
HRBL	HIV-1 Rev-binding protein-like protein	2	9.28	48963	Biological_process unknown
HOMER1	Homer homolog 1	2	5.33	40275	Cell communication ; Signal transduction
HUNK	Hormonally up-regulated neu tumor-associated kinase	2	9.24	79686	Cell communication ; Signal transduction
HSP90AA1	HSP90AA1 protein [Fragment]	2	5.08	73827	Protein metabolism
TTC9C	Hypothetical protein MGC29649.- Homo sapiens (Human).	2	9.02	20013	Biological_process unknown
KPNB1	Importin subunit beta-1	2	4.68	97170	Transport
INA	Internexin neuronal intermediate filament protein, alpha	2	5.34	55391	Cell growth and/or maintenance
IQSEC1	IQ motif and Sec7 domain-containing protein 1	2	6.49	108314	Cell communication ; Signal transduction
KIF17	Kinesin-like protein KIF17	2	5.38	115114	Cell growth and/or maintenance
KIF2A	Kinesin-like protein KIF2A	2	6.28	79955	Cell growth and/or maintenance
L1CAM	L1 cell adhesion molecule [Fragment]	2	5.84	139772	Biological_process unknown
GLO1	Lactoylglutathione lyase	2	5.12	20778	Metabolism ; Energy pathways
LANCL2	LanC lantibiotic synthetase component C-like 2	2	7.18	50850	Biological_process unknown
LSS	Lanosterol synthase	2	6.16	83309	Metabolism ; Energy pathways
CD47	Leukocyte surface antigen CD47 [Precursor]	2	6.82	35214	Immune response
LMO7	LIM domain 7	2	7.90	153670	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
LIN7C	Lin-7 homolog C	2	8.52	21834	Cell communication ; Signal transduction
MDH2	Malate dehydrogenase	2	8.92	35559	Metabolism ; Energy pathways
MTNR1B	Melatonin receptor MEL1B [Fragment	2	9.54	14649	Cell communication ; Signal transduction
ALDH6A1	Methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial [Precursor]	2	8.72	57840	Metabolism ; Energy pathways
MAP1S	Microtubule-associated protein 1S	2	7.21	112437	Cell growth and/or maintenance
RHOT1	Mitochondrial Rho GTPase 1	2	5.87	70784	Apoptosis
MSN	Moesin	2	6.08	67820	Cell growth and/or maintenance
CRYM	Mu-crystallin homolog	2	5.06	33776	Osmoregulation ; Hormone metabolism
MSH5	MutS protein homolog 5	2	5.95	92875	Cell growth and/or maintenance
MYO1D	Myosin-Id	2	9.44	116202	Cell growth and/or maintenance
NDUFB6	NADH dehydrogenase (Ubiquinone) 1 beta subcomplex, 6, 17kDa	2	9.52	13716	Energy pathways ; Metabolism
NDUFB5	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 5, mitochondria	2	9.62	21750	Metabolism ; Energy pathways
NDUFV2	NDUFV2 protein	2	8.22	27349	Metabolism ; Energy pathways
PPP1R9B	Neurabin-2	2	4.91	89192	Cell communication ; Signal transduction
NRXN1	Neurexin-1-alpha [Precursor]	2	5.61	161883	Cell recognition
NF2	Neurofibromin-2	2	6.11	69690	Cell growth and/or maintenance
FREQ	Neuronal calcium sensor 1	2	4.71	21879	Cell communication ; Signal transduction
NRCAM	Neuronal cell adhesion molecule [Precursor]	2	5.45	143894	Signal transduction

NONO	Non-POU domain-containing octamer-binding protein	2	9.01	54232	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
NSFL1C	NSFL1 cofactor p47	2	4.99	40573	Cell growth and/or maintenance
Rotavirus gene-7	NSP3 or NS34	2	5.32	36212	Biological_process unknown
NUCKS	NUCKS protein	2	4.75	18747	Biological_process unknown
NUDC	Nuclear migration protein nudC	2	5.27	38243	Cell communication ; Signal transduction
NUDCD3	NudC domain-containing protein 3	2	5.16	40822	Biological_process unknown
OPTN	Optineurin	2	5.12	65922	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
RP11-632C17__A.1-001	OTTHUMP00000017090 [Fragment]	2	11.00	18712	Biological_process unknown
ODF1	Outer dense fiber protein 1	2	8.46	28366	Cell growth and/or maintenance
OGDHL	Oxoglutarate dehydrogenase-like	2	6.23	114496	Biological_process unknown
P140	p130Cas-associated protein	2	9.32	112466	Biological_process unknown
PPT1	Palmitoyl-protein thioesterase 1 [Fragment]	2	6.94	12131	Metabolism ; Energy pathways
PCNT	Pericentrin (Kendrin)	2	5.39	378081	Cell growth and/or maintenance
PRDX2	Peroxiredoxin-2	2	5.66	21892	Metabolism ; Energy pathways
FARSA	Phenylalanyl-tRNA synthetase alpha chain	2	7.31	57564	Protein metabolism
PHACTR1	Phosphatase and actin regulator 1	2	6.51	66308	Metabolism ; Energy pathways
ATP2B1	Plasma membrane calcium-transporting ATPase 1	2	5.73	138755	Transport
PLXNB3	Plexin-B3 [Precursor]	2	5.96	206847	Cell communication ; Signal transduction
PHB2	Prohibitin-2	2	9.83	33296	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
PCCA	Propionyl-CoA carboxylase alpha subuni	2	7.24	80059	Metabolism ; Energy pathways
BSN	Protein bassoon	2	7.28	416499	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
GBAS	Protein NipSnap2	2	9.42	33743	Cell communication ; Signal transduction
RUFY3	Protein RUFY3	2	5.36	52965	Biological_process unknown
SEC31A	Protein transport protein Sec31A	2	6.43	133015	Transport
PTK2B	PTK2B protein tyrosine kinase 2 beta	2	5.91	115907	Signal transduction
Q5U0P9_HUMAN	Purine-rich element binding protein A	2	6.28	34911	Biological_process unknown
NPEPPS	Puromycin-sensitive aminopeptidase	2	5.49	103276	Protein metabolism
GPCR	Putative G-protein coupled receptor	2	9.14	23857	Biological_process unknown
PHTF1	Putative homeodomain transcription factor 1 [Fragment]	2	9.99	12332	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
DNAJC6	Putative tyrosine-protein phosphatase auxilin	2	6.82	99997	Protein metabolism
CJE0194	Putative uncharacterized protein	2	9.03	23159	Biological_process unknown
Q6PG47_HUMAN	Putative uncharacterized protein [Fragment]	2	9.05	36977	Biological_process unknown
DKFZp451F1711	Putative uncharacterized protein DKFZp451F1711 [Fragment]	2	5.11	93414	Biological_process unknown
DKFZp686D10126	Putative uncharacterized protein DKFZp686D10126 [Fragment]	2	4.77	14872	Biological_process unknown
DKFZp686M09125	Putative uncharacterized protein DKFZp686M09125 [Fragment]	2	4.98	108618	Biological_process unknown
DPP6	Putative uncharacterized protein DPP6 [Fragment]	2	6.11	64982	Transport
RIMS1	Regulating synaptic membrane exocytosis protein 1	2	9.68	189073	Transport
RLBP1	Retinaldehyde-binding protein 1	2	4.98	36474	Transport
RNH1	Ribonuclease inhibitor	2	4.71	49973	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
RPS27	Ribosomal protein S27	2	6.71	7356	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
RBMXL1	RNA binding motif protein, X-linked-like 1 [Fragment]	2	10.30	16850	Biological_process unknown
RSBN1L	Round spermatid basic protein 1-like protein	2	8.92	94368	Biological_process unknown
Q8IWL4_HUMAN	SCCA-PD variant	2	5.24	20161	Biological_process unknown
DKFZp686F17268	Septin	2	8.76	50263	GTPase activity
SEPT2	Septin-2	2	6.15	41487	Cell cycle
SEPT4	Septin-4	2	5.77	55098	Cell cycle
SEPT7	Septin-7	2	8.76	50680	Cell communication ; Signal transduction
SARS	Seryl-tRNA synthetase, cytoplasmic	2	6.05	58777	Metabolism ; Energy pathways
SLC12A5	Solute carrier family 12 member 5	2	5.91	123511	Transport

SORBS1	Sorbin and SH3 domain containing 1	2	6.40	142513	Cytoskeleton organization and biogenesis ; Signal transduction
SKP1A	S-phase kinase-associated protein 1A	2	4.40	18658	Protein metabolism ; Regulation of cell cycle ; Chromosome segregation
SFRS1	Splicing factor, arginine/serine-rich 1	2	10.37	27745	Protein metabolism
SRGAP3	SRGAP3 protein	2	6.20	38869	Cell communication ; Signal transduction
STMN1	Stathmin 1/oncoprotein 18	2	5.76	17337	Cell growth and/or maintenance ; Signal transduction
SLK	STE20-like serine/threonine-protein kinase	2	5.08	142695	Cell communication ; Signal transduction
STUB1	STIP1 homology and U box-containing protein 1	2	5.61	34856	Metabolism ; Energy pathways
STRN	Striatin	2	5.12	86132	Cell communication ; Signal transduction
SAE2	SUMO-activating enzyme subunit 2	2	5.15	71224	Protein metabolism
SOD1	Superoxide dismutase [Cu-Zn]	2	5.70	15936	Metabolism ; Energy pathways
SUGT1	Suppressor of G2 allele of SKP1 homolog	2	5.07	41024	Cell communication ; Signal transduction
SYBU	Syntabulin	2	5.87	72388	Transport
CCT1	T-complex protein 1 subunit alpha	2	5.80	60344	Protein metabolism
TFAM	TFAM protein [Fragment]	2	9.74	29083	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
TXNL1	Thioredoxin-like protein 1	2	4.84	32251	Metabolism ; Energy pathways
TALDO1	Transaldolase	2	6.36	37540	Metabolism ; Energy pathways
TCEAL3	Transcription elongation factor A protein-like 3	2	4.85	22502	Biological_process unknown
PURA	Transcriptional activator protein Pur-alpha	2	6.07	34911	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
GATAD2A	Transcriptional repressor p66 alpha	2	9.95	68063	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
TRIM2	Tripartite motif-containing protein 2	2	6.51	81530	Biological_process unknown
TMOD2	Tropomodulin-2	2	5.21	39595	Cell growth and/or maintenance
TSC22D4	TSC22 domain family protein 4	2	6.69	41026	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
TPPP3	Tubulin polymerization-promoting protein family member 3	2	9.19	18985	Cell growth and/or maintenance
TBCA	Tubulin-specific chaperone A	2	5.25	12855	Protein metabolism
TBCE	Tubulin-specific chaperone E	2	6.32	59346	Protein metabolism
SIRPA	Tyrosine-protein phosphatase non-receptor type substrate 1	2	6.29	54813	Cell communication ; Signal transduction
YARS	Tyrosyl-tRNA synthetase, cytoplasmic	2	6.61	59143	Metabolism ; Energy pathways
LSM3	U6 snRNA-associated Sm-like protein LSm3	2	4.58	11845	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
UNC45B	UNC45 homolog B	2	8.03	103733	Cell growth and/or maintenance
C2orf32	Uncharacterized protein C2orf32	2	7.72	18648	Biological_process unknown
C6orf174	Uncharacterized protein C6orf174 [Precursor]	2	5.81	103199	Biological_process unknown
CXorf23	Uncharacterized protein CXorf23	2	9.71	83871	Biological_process unknown
KIAA1279	Uncharacterized protein KIAA1279	2	5.34	71814	Regulation of cell cycle ;Neurogenesis
CAD34790	unnamed protein product	2	4.75	140303	Biological_process unknown
CAD35032	unnamed protein product	2	5.42	44765	Biological_process unknown
CAD35125	unnamed protein product	2	8.23	20711	Biological_process unknown
CAD61740	unnamed protein product	2	9.03	8255	Biological_process unknown
CAD69692	unnamed protein product	2	11.30	33126	Biological_process unknown
CAE90141	unnamed protein product	2	5.09	37545	Biological_process unknown
CAE99428	unnamed protein product	2	5.05	14510	Biological_process unknown
CAF00110	unnamed protein product	2	9.78	17744	Biological_process unknown
CAF01554	unnamed protein product	2	11.55	6642	Biological_process unknown
CAF86112	unnamed protein product	2	5.95	102932	Biological_process unknown
CAG29885	unnamed protein product	2	10.10	19494	Biological_process unknown
C1orf128	UPF0424 protein C1orf128	2	5.47	24178	Biological_process unknown
ATP6V0D1	Vacuolar ATP synthase subunit d 1	2	4.89	40329	Transport
VSNL1	Visinin-like protein 1	2	5.01	22142	Cell communication ; Signal transduction
WDR1	WD repeat-containing protein 1	2	6.17	66194	Biological_process unknown

Capítulo 7:

Análise Comparativa do Proteoma de Córtex Pré-Frontal Cerebral de Pacientes com Esquizofrenia e Controles Utilizando **Shotgun Proteomics**.

Neste capitulo apresentamos preliminarmente através de uma tabela as proteínas diferencialmente expressas do proteoma de córtex pré-frontal de pacientes com esquizofrenia e controles por *Shotgun Proteomics*.

A rotina de trabalho por *Shotgun Proteomics* descrita na Figura 6 da introdução desta tese foi aplicada às amostras de córtex pré-frontal de pacientes com SCZ e controles para comparação dos proteomas.

Nesta análise pudemos identificar 1261 proteínas, sendo que 106 proteínas apresentaram diferença significativa de expressão. Destas 106 proteínas, 11 tem expressão diminuída enquanto que 95 têm expressão aumentada nos cérebros SCZ. Parte destas proteínas (96) foram agrupadas em 11 classes biológicas funcionais diferentes, sendo as mais representativas as classes de transdução de sinal, manutenção celular e metabolismo de energia, enquanto que 10 proteínas ainda não se encaixam em grupos funcionais conhecidos. A Tabela 1 mostra as proteínas reguladas e suas classes funcionais.

Estes dados ainda estão sendo trabalhados e devem fazer parte de um outro artigo, ainda em fase de elaboração. No entanto, achamos relevante apresentá-los brevemente, devido a comparações que podem ser feitas com os demais dados de transcriptoma e proteoma mostrados nos outros capítulos.

Tabela 1: Proteínas diferencialmente expresssas entre córtex pré-frontal de pacientes com esquizofrenia e controles obtidas por *Shotgun Proteomics* divididas por seus processos biológicos.

Processos Biológicos	*Reg. em SCZ*	*Razão H/L and DP*	*Nome do Gene*	*Nome da Proteína (HPRD)*	*MW (t)*	*Locus Cromos.*	*Pepídeos. Identificados*	*MASCOT Score*
Signal transduction/Cell communication	↓	0.44 +/- 0.00	NME2	Nucleoside diphosphate kinase B *	30137	17q21.3	1	59.59
*** Protein modification*	↓	0.59 +/- 0.00	GPR37	G protein coupled receptor 37	67114	7q31	1	42.12
	↓	0.32 +/- 0.00	CDC42BPG	CDC42 binding protein kinase gamma (DMPK-like)	172518	11q13.1	1	47.17
	↓	0.39 +/- 0.00	SIRPA	SIRP alpha 1	54967	20p13	2	125.11
	↑	2.20 +/- 0.00	STRN	Striatin calmodulin binding protein	86132	2p22-p21	2	44.18
	↑	2.00 +/- 0.00	CHP	Calcium binding protein P22	22456	15q13.3	2	51.92
	↑	1.98 +/- 0.00	CSNK2A1	** Casein kinase II, alpha 1	45143	20p13	2	43.43
	↑	1.65 +/- 0.00	PRKCG	Protein kinase C, gamma	78448	19q13.4	3	61.91
	↑	1.61 +/- 0.00	SIRPB1	Signal regulatory protein beta 1	43254	20p13	3	106.8
	↑	1.60 +/- 0.00	HOMER1	Homer neuronal immediate early gene	40277	5q14.2	2	73.93
	↑	1.55 +/- 0.00	SLC9A3R1	Solute carrier family 9, isoform A3, regulatory factor 1	38868	17q25.1	3	38.88
	↑	1.54 +/- 0.00	MAPK4	Mitogen activated protein kinase 4	62623	18q12-q21	1	47.23
	↑	1.53 +/- 0.00	SH3GL2	SH3 containing GRB2 like protein 2	39962	9p22	5	65.91
	↑	1.52 +/- 0.00	CTNND2	Delta catenin	132656	5p15.2	2	58.55
	↑	1.52 +/- 0.00	OPA1	Dynamin like 120 kDa protein, mitochondrial	117770	3q28-q29	4	73.1
	↑	1.49 +/- 0.00	GSPT1	G1 to S phase transition 1	55756	16p13.1	2	65.64
	↑	1.47 +/- 0.00	TBC1D24	TBC1 domain family, member 24	62318	16p13.3	1	44.93
	↑	1.46 +/- 0.00	PPP1R9B	Protein phosphatase 1, regulatory subunit 9B	89451	17q21.33	2	56.27
	↑	1.43 +/- 0.00	SORBS1	Sorbin and SH3 domain containing 1	142496	10q23.3-q24.1	2	42.14
	↑	1.42 +/- 0.00	PEBP1	Phosphatidylethanolamine binding protein (Raf kinase inhibitor protein)	21057	12q24.23	19	51.19
	↑	1.41 +/- 0.00	PRKCE	Protein kinase C epsilon	83674	2p21	1	120.52
	↑	1.40 +/- 0.00	GARNL4	GTPase activating Rap/RanGAP domain like 4	80042	17p13.3	1	54.08
Cell growth/maintenance	↓	0.53 +/- 0.00	MYO1D	Myosin ID	116202	17q11-q12	2	76.37
	↑	3.01 +/- 0.00	KRT1	Keratin 1	66067	12q12-q13	1	39.83
	↑	1.86 +/- 0.00	VIL2	Ezrin	69413	6q25.2-q26	1	58.52
	↑	1.70 +/- 0.00	NSFL1C	NSFL1 (p97) cofactor (p47)	40573	20p13	2	44.69
	↑	1.67 +/- 0.00	VIM	Vimentin	53652	10p13	2	74.17
	↑	1.64 +/- 0.00	KIF21A	Kinesin family member 21A	185510	12q12	2	47.63
	↑	1.62 +/- 0.00	LMNB2	Lamin B2	67689	19p13.3	4	47.66
	↑	1.58 +/- 0.00	MAP1A	Microtubule associated protein 1A	291964	15q13-qter	5	50.55
	↑	1.58 +/- 0.00	TUBB2B	Tubulin beta polypeptide paralog	49953	6p25	7	98.31
	↑	1.53 +/- 0.00	MAP2	Microtubule associated protein 2	202758	2q34-q35	7	94.94
	↑	1.52 +/- 0.00	TRIM3	Brain expressed RING finger	80829	11p15.5	2	78.65
	↑	1.52 +/- 0.00	MAP6	Microtubule associated protein 6	86505	11q13.3	3	41.03
	↑	1.52 +/- 0.00	MRLC2	Myosin regulatory light chain MRLC2	19779	18p11.31	2	65.91
	↑	1.46 +/- 0.00	EPB41L1	Neuron type nonerythroid protein 4.1	98503	20q11.2-q12	2	59.57
	↑	1.42 +/- 0.00	DES	Desmin	53536	2q35	2	75.79
	↑	1.41 +/- 0.00	EPB41L3	Band 4.1 like protein 3	120677	18p11.32	7	84.97
	↑	1.40 +/- 0.00	PKP4	Plakophilin 4	131868	2q23-q31	2	39.5
Metabolism/Energy pathways	↓	0.60 +/- 0.00	CS	Citrate synthase	51712	12q13.2-q13.3	1	93.17
	↓	0.50 +/- 0.00	CNP	2`,3` cyclic nucleotide,3`-phosphodiesterase	45099	17q21	18	93.81
	↑	3.69 +/- 0.00	PRDX6	Peroxiredoxin 6	25035	1q25.1	4	55.98

	↑	2.47 +/- 0.00	AK1	Adenylate kinase 1	21635	9q34.1	3	50.9
	↑	1.78 +/- 0.00	PPT1	Palmitoyl protein thioesterase 1	34193	1p32	2	41.11
	↑	1.71 +/- 0.00	CKB	Creatine kinase brain type	42644	14q32	5	86.64
	↑	1.68 +/- 0.00	PFKM	Phosphofructokinase	85182	12q13.3	2	52.28
	↑	1.56 +/- 0.00	NDUFV2	NADH ubiquinone oxidoreductase flavoprotein 2	27363	18p11.31-p11.2	2	76.63
	↑	1.55 +/- 0.00	UQCRC1	Cytochrome bc1	52646	3p21.3	1	94.36
	↑	1.53 +/- 0.00	HK1	Hexokinase 1	102738	10q22	2	62.27
	↑	1.44 +/- 0.00	PRDX2	Peroxiredoxin 2	21892	19p13.2	2	90.83
	↑	1.42 +/- 0.00	STUB1	STIP1 homologous and U box containing protein 1	34856	16p13.3	2	86.17
	↑	1.41 +/- 0.00	GAPDH	Glyceraldehyde 3 phosphate dehydrogenase	36054	12p13	25	87.78
	↑	1.40 +/- 0.00	ADH5	Alcohol dehydrogenase 5, chi polypeptide	39724	4q21-q25	2	57.78
Regulation of nucleobase, nucleoside,	↑	1.96 +/- 0.00	HNRPA0	Heterogeneous nuclear ribonucleoprotein A0	30841	5q31	2	62.9
nucleotide and nucleic acid metabolism	↑	1.92 +/- 0.00	BSN	Zinc finger protein 231	416464	3p21.31	2	59.78
**:also belongs to this class*	↑	1.90 +/- 0.00	HP1BP3	HP1-BP74	61207	1p36.12	2	51.2
	↑	1.83 +/- 0.00	NUCKS1	Nuclear ubiquitous casein kinase and cyclin dependent kinase substrate	27296	1q32.1	2	144.54
	↑	1.82 +/- 0.00	THRAP3	TRAP150 (thyroid hormone receptor associated protein 3)	108694	1p34.3	1	53.19
	↑	1.69 +/- 0.00	PurA	Purine rich element binding protein A	34911	5q31	2	54.29
	↑	1.56 +/- 0.00	HNRPK	Heterogeneous nuclear ribonucleoprotein K	51028	9q21.32-q21.33	3	83.54
	↑	1.53 +/- 0.00	SFRS6	Splicing factor, arginine/serine-rich 6	39587	20q12-q13.1	3	43.85
	↑	1.49 +/- 0.00	FUBP1	Far upstream element binding protein	68604	1p31.1	2	47.36
	↑	1.46 +/- 0.00	RBM9	RNA binding motif protein 9	47236	22q13.1	1	41.38
	↑	1.45 +/- 0.00	NONO	Non pou domain containing octamer binding protein	54231	Xq13.1	2	71.75
	↑	1.44 +/- 0.00	SFRS7	Splicing factor, arginine/serine-rich 7,35kDa	27366	2p22.1	2	94.2
	↑	1.42 +/- 0.00	HNRPM	Heterogeneous nuclear ribonucleoprotein M	77515	19p13.3-p13.2	1	51.78
	↑	1.42 +/- 0.00	HNRPU	Heterogeneous nuclear ribonucleoprotein U	90584	1q44	5	45.76
Protein metabolism	↓	0.54 +/- 0.00	HSPA5	BIP	72333	9q33-q34.1	2	81.99
	↑	1.84 +/- 0.00	FGB	Fibrinogen, beta chain	55928	4q28	1	38.49
	↑	1.61 +/- 0.00	UBE2N	Ubiquitin conjugating enzyme E2N	17138	12q22	3	97.82
	↑	1.53 +/- 0.00	CCT3	Chaperonin containing T complex polypeptide 1, subunit 1	60534	1q23	2	93.78
	↑	1.50 +/- 0.00	DNAJC6	DnaJ (Hsp40) homolog subfamily C member 6	99996	1pter-q31.3	2	76.4
	↑	1.49 +/- 0.00	FARSLA	Phenylalanyl-tRNA synthetase alpha chain	57563	19p13.2	2	44.11
	↑	1.46 +/- 0.00	SERPINA3	Alpha 1 antichymotrypsin	47651	14q32.1	2	41.1
	↑	1.46 +/- 0.00	HSPA1A	Heat shock 70 KD protein 1A	70038	6p21.3	10	94.04
	↑	1.41 +/- 0.00	CKAP1	Cytoskeleton associated protein 1	27325	19q13.11-q13.12	4	66.94
Transport	↓	0.54 +/- 0.00	HBB	Hemoglobin beta chain	15998	11p15.5	7	56.38
	↑	1.72 +/- 0.00	RLBP1	Retinaldehyde binding protein 1	36474	15q26	2	51
	↑	1.66 +/- 0.00	ATP6V0D1	ATPase H+ transporting lysosomal 38 KD V0 subunit D, isoform 1	40329	16q22	2	89.36
	↑	1.51 +/- 0.00	APOE	Apolipoprotein E	36154	19q13.2	2	43.33
	↑	1.50 +/- 0.00	VAPB	VAMP associated protein B	27228	20q13.33	5	59.09
	↑	1.47 +/- 0.00	ATCAY	Caytaxin	42120	19p13.3	2	53.87
Immune response	↓	0.31 +/- 0.00	MOG	Myelin oligodendrocyte glycoprotein	28647	6p22.1	1	97.74
	↓	0.57 +/- 0.00	IGSF4B	Immunoglobulin superfamily member 4B	47021	1q21.2-q22	2	66.04
	↑	1.69 +/- 0.00	KIR3DL2	NK associated transcript 4	50216	19q13.4	2	109.11
Cytoskeletal anchoring	↑	1.68 +/- 0.00	PLEC1	Plectin 1	531789	8q24	2	46.48

Osmoregulation/Hormone metabolism	↑	1.56 +/- 0.00	CRYM	Crystallin mu	33775	16p13.11-p12.3	2	106.46
DNA repair	↑	1.89 +/- 0.00	APEX2	DNA-(apurinic or apyrimidinic site) lyase 2	57400	Xp11.22	2	56.43
Biological_process unknown	↑	2.92 +/- 0.00	CGI-38	CGI-38 brain specific protein	18985	16q22.1	2	94.3
	↑	1.88 +/- 0.00	WDR1	WD repeat protein 1	66193	4p16.1	2	55.84
	↑	1.59 +/- 0.00	EFHD2	EF hand domain family member D2	26697	1p36.21	5	53.89
	↑	1.54 +/- 0.00	RBMXL1	Similar to RNA binding motif protein X linked	42141	1p22.2	2	74.47
	↑	1.51 +/- 0.00	KIAA1189	Hypothetical protein KIAA1189 (Ermin (myelinating oligodendrocyte-specific protein))	34301	2q24.1	2	78.22
	↑	1.49 +/- 0.00	LANCL2	LanC lantibiotic synthetase component C like 2	50854	7q31.1-q31.33	2	48.03
	↑	1.45 +/- 0.00	L1CAM	L1 cell adhesion molecule	140002	Xq28	2	71.6
	↑	1.43 +/- 0.00	RTN4	Reticulon 4	129931	2p16.3	5	40.83
	↑	1.42 +/- 0.00	TTC9C	Hypothetical protein MGC29649	20013	11q12.3	2	41.67
	↑	1.41 +/- 0.00	SEPT4	Septin 4	55098	17q22-q23	2	53.71

Conclusões:

O intuito principal deste projeto, ao realizar estudos comparativos de expressão global gênica e protéica em amostras SCZ e CTRL, foi identificar alterações moleculares consistentes que permitissem avançar no conhecimento da esquizofrenia.

Apesar desta idéia clara e bem definida, na prática a busca de um marcador molecular consistente para uma doença complexa como a esquizofrenia não é uma tarefa simples, por diversos motivos. A princípio, a maior limitação é a obtenção de material biológico útil para estas análises. Idealmente é necessário trabalhar com amostras cerebrais (obtidas por necrópsia), de pacientes que geralmente foram diagnosticados há muitos anos (em geral na adolescência) e receberam tratamento crônico com anti-psicóticos variados normalmente durante toda a vida adulta. Deste modo, torna-se extremamente complexa a tarefa de, ao identificar um marcador potencial, determinar se este é uma causa ou uma consequência da doença, ou se este é fruto de alterações causadas pelos medicamentos usados pelos pacientes, ou ainda se é resultado das variações individuais outras, não relacionadas com a doença. Neste contexto, a obtenção de amostras cerebrais de pacientes com pequena variação de sua história clínica, em quantidade adequada e qualidade suficiente e ainda pareadas com amostras da mesma região cerebral, derivadas de indivíduos controle, pareados por sexo e idade, mas sem antecedentes psiquiátricos, torna o processo muito desafiador. Deste modo, somos extremamente gratos ao grupo da Dra. Andrea Schmitt, que viabilizou a condução deste projeto, ao nos permitir acesso a um importante universo amostral, adequadamente pareado.

Ao passarmos por todas estas limitações, e finalmente gerarmos os dados, é fundamentel buscar uma validação dos achados. Após avaliarmos a expressão de mais de 20 mil transcritos, usando SAGE, identificamos diversos potenciais marcadores transcricionais em SCZ. Boa parte dos achados significativos de SAGE pôde ser confirmada por dados de outros grupos, que apontaram alterações nos

mesmos genes, mesmo usando amostras e técnicas distintas das que empregamos em nosso estudo. Por outro lado, as análises de SAGE demonstraram um novo universo de marcadores, ainda inéditos. Uma fração destes foi avaliada por qPCR. Dos 4 genes avaliados, três demonstraram uma clara redução de expressão em SCZ, de modo coerente com o observado por SAGE. Em dois dos 4 genes, a diferença de expressão entre controles e SCZ foi estatisticamente significativa.

Os estudos de proteoma conduzidos tanto por eletroforese quanto por *Shotgun* possibilitaram a identificação de aproximadamente 120 proteínas diferencialmente expressas diferentes em duas regiões cerebrais distintas inéditas em estudos de proteoma de SCZ. Nossos dados reforçaram a diferença de expressão de algumas proteínas reveladas em trabalhos anteriores em diferentes regiões cerebrais, além de apontar inéditos potenciais biomarcadores, que devem ser confirmados em amostras individuais.

Apesar dos dados de transcriptoma e proteoma de córtex pré-frontal sugerirem a regulação diferencial das mesmas vias bioquímicas e revelarem certa correspondência entre os dados como a diferença de expressão gênica e protéica de MBP, ALDO, ATP5A1, HSPC e DNM1, não conseguimos observar um agrupamento coeso entre os dois conjuntos de dados (transcriptoma e proteoma), visto que a correspondência de genes e proteínas diferencialmente expressas não foi tão evidente. Entretanto, isso tem sido observado em diversos projetos de transcriptoma e proteoma comparativos, o que aumentou as discussões sobre o estabelecimento de alvos moleculares[1]. Por mais que a idéia do estabelecimento de biomarcadores seja simples e sustentável, a atual prática desta idéia tem mostrado que:

* I) Alterações na expressão gênica nem sempre resultam em alterações da expressão da proteína e alterações protéicas nem sempre são derivadas de alterações transcricionais.

* II) As diferenças entre as tecnologias disponíveis, principalmente devido a diferença da estrutura molecular de ácidos nucléicos e proteína, limitam as

[1] Mischak H, Apweiler R, Banks RE, Conaway M, Coon J, Dominiczak A, Ehrich JHH, Fliser D, Girolami M, Hermjakob H, Hochstrasser D, Jankowski J, Julian BA, Kolch W, Massy ZA, Neususess C, Novak J, Peter K, Rossing K, Schanstra J, Semmes OJ, Theodorescu D, Thongboonkerd V, Weissinger EM, Van Eyk JE, Yamamoto T. Clinical proteomics: A need to define the field and to begin to set adequate standards. Proteomics Clin. Appl. Vol 1, (2): 148-156.

comparações entre transcriptoma e proteoma. Enquanto as análises de ácidos nucléicos têm plataformas mais bem estabelecidas e maior sensibilidade permitindo uma análise de quase todo o transcriptoma, além de um banco de dados mais amplo por conta dos projetos de sequenciamento em larga-escala, a proteômica ainda encontra-se limitada a uma análise da fração mais abundante das proteínas expressas, mesmo considerando técnicas mais recentes como *Shotgun Proteomics* que visam aumentar a sensibilidade. Os métodos utilizados neste trabalho (SAGE, qPCR, 2-DE, MS e *Shotgun*) são bem distintos, com vieses particulares que devem, de fato, apontar universos diferentes de marcadores moleculares.

* III) A complexidade bioquímica dos organismos nos impossibilita acreditar que exista um marcador único para determinada doença. Se uma dada via metabólica encontra-se deficitária, é plausível supor que alterações de outros pontos desta via gerem os mesmos efeitos, e resultem no desenvolvimento da mesma doença. É possível ainda que uma coleção de pequenas alterações, em genes ou proteínas, tais como variações de expressão que não tenha significado estatístico, em conjunto resultem em uma patologia. Ainda, há os que defendem que não existe um marcador molecular específico para cada doença, visto a complexidade de interações entre genes e proteínas.

* IV) as diferenças intrínsecas de cada indivíduo analisado (seja ele um controle ou um paciente), principalmente em se tratando de amostras de tecido humano, podem revelar também diferenças de expressão gênica e protéica não relacionadas à doença e sim a fatores ambientais. Ainda, as diferenças moleculares peculiares a cada pessoa provinda de sua individualidade genômica podem interferir na expressão de seus genes e proteínas, de modo independente da existência ou não de uma dada condição patológica.

Reduzir as diferenças apontadas no ultimo parágrafo foi um dos motivos principais para a confecção de *pools*: a rigor, o estudo de *pools* de amostra reduziria as diferenças de expressão gênica e protéica intrínsecas de cada amostra, resultado das interferências do ambiente e das peculiaridades bioquímicas dos pacientes, ressaltando as diferenças de expressão gênica e

protéica que se referem a uma característica comum a todos os indivíduos do *pool*: seu *status* como controle ou paciente. Contudo, como pudemos observar em nossos experimentos de SAGE e qPCR, alterações muito grandes de expressão gênica (ou protéica) como a ocorrida em 2 das 9 amostras para o gene MALAT1 podem parecer ser comuns às amostras do *pool*, quando na verdade não são. Nossos resultados nos fizeram repensar os prós e contras do estudo de *pool* e a validade dos seus achados.

Apesar de toda a problemática abordada em relação ao estabelecimento de marcadores moleculares, as análises globais como desenvolvidas neste projeto puderam apontar para pelo menos uma via consistentemente alterada na SCZ; nossos dados de transcriptoma e proteoma foram sólidos e confirmaram alterações significativas no metabolismo de oligodendrócitos. No entanto, o conceito de que os sintomas da SCZ podem ser resultado da decomposição ou da regulação diferencial de oligodendrócitos devem ser visto com cautela. O uso crônico de haloperidol, uma das drogas mais usadas no tratamento da SCZ, parece levar a uma redução na expressão de genes relacionados a oligodendrócitos[2]. Porém, fortes evidências a partir de diversas linhas de pesquisa como dados robustos de expressão gênica e técnicas de imagem[revisado em 3] apontam o potencial envolvimento direto dos oligodendrócitos na SCZ.

Nossos dados puderam confirmar também, através da expressão protéica diferencial de diversas proteínas do complexo I mitocondrial e através da expressão gênica diferencial de subunidades de ATPase mitocondrial que o déficit do metabolismo energético pode causar (ou ser conseqüência) da SCZ. No entanto, cabe uma nota de cautela, pois já foi observado que enzimas metabólicas como a malato desidrogenase foram encontradas reguladas no hipocampo de ratos tratados com clorpromazina e clozapina[4].

Nossos dados ressaltaram consistentemente que a homeostase do íon cálcio está alterada tanto no lobo temporal anterior quanto no córtex pré-frontal

[2] Narayan S, Kass KE, Thomas EA. Chronic haloperidol treatment results in a decrease in the expression of myelin/oligodendrocyte-related genes in the mouse brain. J Neurosci Res. 2007; 85(4):757-765.
[3] Segal D, Koschnick JR, Slegers LH, Hof PR. Oligodendrocyte pathophysiology: a new view of schizophrenia.Int J Neuropsychopharmacol. 2007 Aug;10(4):503-11.
[4] La Y, Wan C, Zhu H, Yang Y, Chen Y, Pan Y, Ji B, Feng G, He L. Hippocampus protein profiling reveals aberration of malate dehydrogenase in chlorpromazine/clozapine treated rats. Neurosci Lett. 2006 Nov 6;408(1):29-34.).

dos pacientes SCZ. Acredita-se que alguns dos sintomas da SCZ podem ser consequência de uma comunicação debilitada entre diferentes regiões cerebrais. Esta comunicação deficitária pode ser causa ou consequência não somente de fatores de neurodesenvovimento, mas também de neurotransmissão, que tem ligação direta com a geração e transmissão do impulso nervoso, e portanto com a homeostase do íon cálcio. Além disso, o íon cálcio tem vital importância para a função dos receptores de dopamina[5], além de influenciar na atividade de diversas enzimas relacionadas à função da membrana neuronal, tais como as fosfolipases do tipo A2 (PLA2), que foram consistentemente encontradas alteradas em amostras de pacientes com SCZ[6,7].

Ainda, nossos dados mostraram que genes codificadores de proteínas cuja classe funcional ainda é desconhecida (Tabela 2 do Capítulo 1), além de proteínas hipotéticas (Tabelas das proteínas alteradas dos Capítulos 4, 5 e 6) podem estar envolvidas com esta patologia.

Deste modo, ao gerarmos estes dados, acreditamos ter contribuído com o melhor conhecimento dos mecanismos moleculares envolvidos com a esquizofrenia e esperamos ter apontado novos marcadores moleculares consistentes.

[5] Bergson C, Levenson R, Goldman-Rakic PS, Lidow MS. Dopamine receptor-interacting proteins: the Ca(2+) connection in dopamine signaling. Trends Pharmacol Sci. 2003; 24(9):486-492.
[6] Gattaz WF, Kollisch M, Thuren T, Virtanen JA, Kinnunen PK. Increased plasma phospholipase-A2 activity in schizophrenic patients: reduction after neuroleptic therapy. Biol Psychiatry. 1987;22(4):421-426.
[7] Barbosa NR, Junqueira RM, Vallada HP, Gattaz WF. Association between BanI genotype and increased phospholipase A2 activity in schizophrenia.Eur Arch Psychiatry Clin Neurosci. 2007 Sep;257(6):340-3.

Perspectivas:

Os dados resultantes de nosso trabalho representam nosso esforço no sentido de contribuir para a maior compreensão da SCZ, gerando uma lista de potenciais marcadores moleculares – transcricionais e protéicos - para esta doença. A validação de nossos achados dependerá de análises subsequentes em amostras individualizadas e oriundas de um maior número de pacientes, de modo a cobrir os importantes aspectos de variações individuais e endofenótipos.

Genes diferencialmente expressos de classes funcionais desconhecidas e proteínas hipotéticas diferencialmente expressas em SCZ, assim como genes e proteínas já conhecidas mas nunca relacionadas à doença merecem atenção especial, devido seu potencial de desvendar caminhos fisiológicos ainda não imaginados durante a evolução patológica da SCZ. Estes novos marcadores, se robustamente confirmados, podem sinalizar para o tratamento e diagnóstico desta neuropatologia, a partir de abordagens ainda não investigadas.

Esperamos que a publicação dos dados de expressão gênica e protéica global por nós obtidos sirva de alicerce para trabalhos posteriores em diversas áreas do conhecimento como estudos de imagem, além de outros estudos de expressão gênica e protéica. Diante da heterogeneidade funcional das diferentes áreas cerebrais, na SCZ, seria importante definirmos a expressão de marcadores em diferentes áreas do cérebro, validando sua consistência ou mesmo a sua diversidade. A explicação da expressão diferencial que observamos poderá ser objeto de futuros estudos, onde investigaríamos desde alterações envolvendo polimorfismos de DNA, passando por experimentos in vitro e in vivo para avaliar o papel funcional dos marcadores observados, até o estudo de microRNAs, moléculas regulatórias que poderiam ter um papel importante e poderiam explicar a origem da expressão diferencial observada, ajudando no entendimento da complexidade das vias bioquímicas envolvidas.

Somos diariamente questionados pelas famílias de pacientes com SCZ sobre a proximidade que nossa pesquisa está da cura da SCZ. Por mais que não seja fácil dizer para estas pessoas, que confiam a nós esta tarefa, que na verdade o nosso trabalho contribui com uma pequena fração desta esperada cura ou mesmo um melhor tratamento para os pacientes, cremos que nossos dados colaboram para uma melhor perspectiva da SCZ.