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Proteomics in Ménière Disease

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Ménière's disease (MD) is a disorder of the inner ear characterized by an insidious onset and aspecific symptoms, such as dizziness, vertigo, tinnitus, and hearing loss, that may become very debilitating. The presence of endolymphatic hydrops is a common feature in MD patients, but the pathophysiology is still largely unknown. In this study, we have used a proteomics-driven approach to identify potential biomarkers of MD. To this end, plasma was obtained from whole blood of 16 individuals previously diagnosed as suffering from MD and compared to plasma from healthy donors. A depletion of the highly abundant proteins (i.e., albumin, IgG, transferrin, etc.) was performed in order to enhance the chance of detection of the less represented ones, therefore reducing the noise-background. Two-dimensional gel electrophoresis, followed by in-gel tryptic digestion of the selected spots and LC-MS/MS analysis, allowed us to identify a set of proteins whose expression appears to be differentially modulated in patients versus controls. In particular: complement factor H and B, fibrinogen alpha and gamma chains, beta-actin and pigment epithelium derived factor are over expressed; on the other hand, the levels of beta-2 glycoprotein-1, vitamin D binding protein and apolipoprotein-1 are significantly decreased in the plasma of MD-affected individuals. Even though preliminary and not necessarily linked directly to the molecular pathogenesis of the disease, our original findings suggest that a molecular signature, represented by the plasma protein profile previously described, might represent a potentially powerful, innovative and not invasive tool for early diagnosis and clinical management of MD patients.

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MD is an inner ear disorder characterized by recurrent episodes of vertigo, fluctuating sensorineural hearing loss, tinnitus, and aural fullness. More frequently affecting one ear, the involvement of the contralateral ear has been reported with a wide range of variability in literature (Balkany et al., 1980). There is no general agreement on the incidence of MD; this is mainly due to the fact that reported studies are based on questionnaires sent to samples of the general population, by using different criteria (Wladislavosky-Waserman et al., 1984; Guilemany et al., 2004; Havia et al., 2005). In medical practice, MD seems to be overdiagnosed, with a rate of confirmation of only 40% (Kotimäki et al., 1999; Neuhauser, 2007). The etiology and pathophysiology of this disease are still under heavy debate. The most descriptive pathologic feature of MD is endolymphatic hydrops, demonstrated from histopathologic studies (Salt and Plontke, 2010), more likely caused by endolymphatic malabsorption at level of endolymphatic duct and sac, with consequent dysfunction of the hydroionic homeostasis and abolition of endocochlear potential (Paparella and Djalilian, 2002). On the other hand, the etiologic basis of the disease is a multifactorial inheritance of intrinsic and extrinsic factors. Even though the American Academy of Otolaryngology-Head and Neck Surgery (AAO-HNS) has published guidelines for diagnosis of MD (Monsell et al., 1995), still there is a lack of consensus about diagnostic criteria: in fact there is no single feature or subset of features from the history, clinical examination or instrumental tests that are able to establish a certain diagnosis. Aim of this study was to identify potential biomarkers of MD using a proteomics-driven approach.

Materials and Methods

Clinico-pathological characteristic of the patients and sample collection

Samples were collected between March 2010 and October 2010 at the Audiology and Phoniatrics Unit, Magna Graecia University, Medical School, Catanzaro, Italy, and officially registered. All patients were asked to give informed written consent approved by the ethical committee of the Medical School.

Blood plasma was obtained from 12 patients (6 men, 6 women, mean age 51 years) affected by definite MD according AAO-HNS guidelines (Monsell et al., 1995) and 8 healthy anonymous donors (pair-matched by sex and age) in according with HUPO plasma proteome guidelines (Rai et al., 2005). Eight to 10 ml of blood were

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TABLE 1. Pools of plasma samples were made for proteomic analysis according to the disease status

Pool number	Menière's disease patients	Healthy donors
1	1–4	
2	5–8	
3	9–12	
4		1–8

drawn by venipuncture and collected in a K_2EDTA tubes. Each sample was subjected to centrifugation (1,300*g* for 10 min) within 2 h of collection and multiple 20 μl aliquots were stored into silicon tubes at −80°C until use.

Study design

Four distinct plasma pools were prepared as described in Table 1. Samples were pre-treated with the ProteomeLab™ IgY-12 Proteome Partitioning Kit (Beckman Coulter, Crea, CA) to remove the 12 highly abundant proteins from plasma [albumin, total IgG, α1-antitrypsin, IgA, IgM, transferrin, haptoglobin, α1-acid glycoprotein (orosmucoid), α2-macroglobulin, HDL (apolipoproteins A-I and A-II) and fibrinogen]. This immunoaffinity procedure provides an enriched pool of lower abundant proteins for downstream proteomic analysis.

Two-dimensional PAGE separation

Plasma samples were thawed and 120 μg of proteins for each pool was acetone-precipitated by overnight incubation at −20°C. Following centrifugation (10,000*g* for 30 min), pellets were resuspended in rehydration buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 50 mM DTT, 0.8% (v/v) ampholytes (pH 3–10) and applied to 24 cm non-linear IPG strips pH 4–7 (GE Healthcare, Little Chalfont, Buckinghamshire, UK). First dimension separation was performed at a constant temperature of 16°C with stepwise increase voltage for the time period necessary to reach for a total of 75,000 Vh in total. The IE-focused strips were subsequently incubated for 15 min with equilibration buffer containing 6 M urea, 20% (v/v) glycerol, 2% (w/v) SDS, 0.05 M Tris/HCl pH 8.8 and 2% DTT. Free thiol groups protein alkylation was performed for 15 min in the same solution containing 2.5% (w/v) iodoacetamide instead of DTT. Second dimension was carried out with an Ettan Daltsix Electroforesis System (GE Healthcare) at 20°C, overnight. Strips were loaded on the top of a 10% SDS–polyacrylamide gel and fixed with agarose. Power was held constant to 2 W/gel until the bromophenol blue line reached the bottom of the gel. Three gels were prepared from each sample, fixed for 2 h in a solution containing 40% ethanol and 10% acetic acid and finally stained with Dodeca™ Silver Stain Kit according to manufacturer's instructions.

Image analysis and in-gel digestion

Silver-stained gels were scanned using an UMAX Magic Scanner (GE Healthcare) at 1,200 dpi resolution and analyzed using the Image Master 2D Platinum software (GE Healthcare). Spots selected for protein identification were excised from gels and washed at room temperature with $K_3Fe(CN)_6$ and $Na_2S_2O_3$. Then gel pieces were washed with NH_4HCO_3 at 37°C. Finally, tryptic digestion was carried out overnight at 37°C as described elsewhere (Shevchenko et al., 2007).

LC-MS/MS analysis

Peptide samples were purified and concentrated using PepClean™ C-18 Spin Columns. Each spin column contains a porous C-18 reversed-phase resin with good binding and recovery characteristics at a wide range of peptide concentrations. PepClean™ C-18 Spin Columns remove interfering contaminants and release peptides in MS-compatible solutions, resulting in increased sensitivity and high-quality spectra.

Peptides were fractionated and fragmented by using a high-performance hybrid quadrupole time-of-flight mass spectrometer, QSTAR® XL Hybrid LC/MS/MS System (Applied Biosystems, Foster City, CA), in positive ionization, with an ESI of 1,800 V, curtain gas 15 unit, CID gas 3 unit. Protein identification was carried out using Mascot search program. Search parameters were as follows: Peptide Mass Tolerance: ±30 ppm; Fragment Mass Tolerance: ±0.8 Da; Variable modifications: Oxidation (M); Enzyme: Trypsin; Max Missed Cleavages: 2; Taxonomy: Homo sapiens.

Statistical analysis

Student's *t*-test was used to identify differences in mean values between the two groups. $P < 0.05$ (two-sided) was considered statistically significant.

Results

Two-dimensional PAGE protein separation and image analysis

Triplicates of 2-DE protein maps were obtained from each pool using 24 cm strips focused in the 4–7 pH range and subsequently separated on a 10% PAGE. Representative gels are shown in Figure 1. The removal of highly abundant plasma proteins prior to profiling allowed a good resolution of the remaining, less represented, peptides.

Image analysis of the gels, carried out using the Image Master 2D-Platinum software, highlighted the presence of several spots differentially expressed in the two subgroups (MD patients vs. healthy donors); on the other hand, no differences were detected among pools 1, 2, and 3, all belonging to the same population (MD-affected individuals).

In-gel digestion and subsequent LC-MS/MS analysis of the excised spots revealed, in MD patients, an increased expression of the following proteins: complement factor H and B, fibrinogen alpha and gamma chains, beta-actin and pigment epithelium derived factor. On the other side, the plasma proteome of MD-affected individuals showed decreased levels of: beta-2 glycoprotein-1, vitamin D binding protein and apolipoprotein-I (Table 2). All differences detected among the proteins showing either up- or downregulation among the two groups were statistically significant. Three additional protein spots, differentially expressed within the two subgroups, did not correspond to any of the known proteins in the available databases. As an example, densitometric analysis of beta-2 glycoprotein and apolipoprotein-I is shown in Figure 2. LC-MS/MS findings on pooled samples were confirmed by performing the analysis on a single patient basis. Further confirmation with an independent assay was obtained by challenging plasma from each of the individuals recruited in the present study with antibodies specifically recognizing the proteins differentially expressed in MD patients by means of Western blotting analysis (data not shown).

Discussion and Conclusions

The pathophysiology of MD is quite complex and heavily debated. It is still unclear, for example, whether the presence of endolymphatic hydrops, histologically documented by post-mortem examination in several cases, is the trigger of cochleovestibular dysfunction or whether hydrops is an epiphenomenon of a more subtle biochemical perturbation that underlies the disease state (Paparella and Djalilian, 2002). A familial predisposition has been reported in about 3–12% of patients with MD but the genetics of the disease is extremely heterogeneous. In an elegant review on the basic science of MD, Semaan et al. (2005) have recently stated that familial MD is transmitted, at least in a subset of patients, in an autosomal dominant fashion, with a variable penetrance and evidence

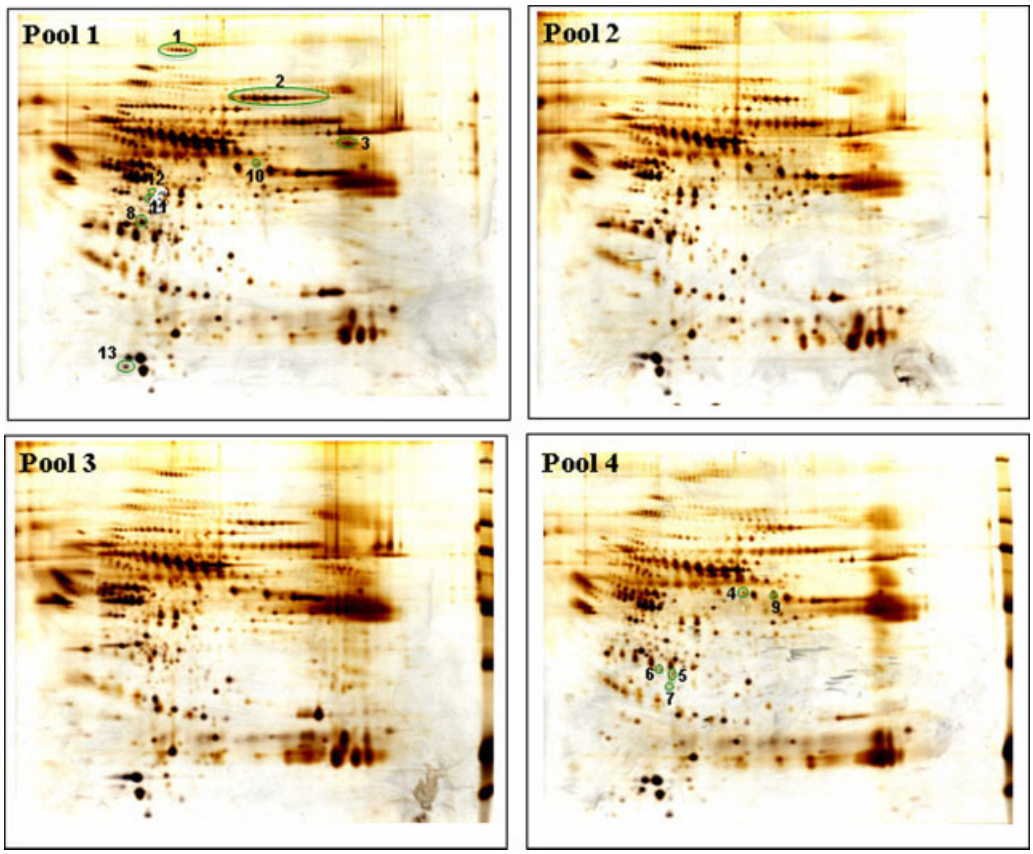

Fig. 1. Silver-stained 2D gels of MD patients and healthy donors. Pool A: patients 1–4; Pool B: patients 5–8; Pool C: patients 9–12; pool D: controls. Twelve spots were differentially expressed when MD samples were compared with non-MD, control samples, by proteomics analysis (green circles).

suggestive of anticipation (i.e., worsening of the clinical phenotype through successive generations). Linkage analysis has not been successful so far in identifying potential candidate genes in familial MD (Morrison and Johnson, 2002); on the other hand, the observation of a close association of this disease with certain HLA antigens (specifically HLA-A3, B7, CW7, and DR2) (Xenellis et al., 1986; Morrison et al., 1994), raises the possibility of an autoimmune pathogenetic basis. In a study aimed at defining the biochemical composition of the "homogeneous substance" that normally occupies the endolymphatic sac lumen but rapidly disappears in response to expansion of the endolymph volume (hallmark of the MD), Thalmann et al. (2006) have found that midmolecular weight, acidic proteins, are the majority of detectable proteins, and that most of these are degraded by deglycosylating enzymes. Overall, the availability of reliable, reproducible, and easy-to-obtain diagnostic markers is urgently needed.

In this study, we have identified a proteomic profile from plasma of MD-affected individuals that can be potentially used as a diagnostic tool for large screening studies in this specific population. Among the identified proteins, we would like to draw the reader's attention on few of them because of their involvement in ear-related disorders. Complement factor H (CFH), is a serum glycoprotein that plays an important role in the alternative complement pathway in fluid phase and on cellular surfaces. Mutations in this protein have been linked to

TABLE 2. List of proteins whose expression is differentially regulated in plasma from MD patients versus healthy donors

Spot number	Protein	Mass	Score	No. of peptides	MD vs. controls
2010-02-01	Complement factor H	143,654	673	29	Upregulated
2010-02-02	Complement factor B	86,847	660	30	Upregulated
2010-02-03	Fibrinogen alpha chain	95,656	320	18	Upregulated
2010-02-04	Beta-2-glycoprotein I	39,584	53	1	Downregulated
2010-02-05	Vitamin D-binding protein	54,526	88	9	Downregulated
2010-02-06	Vitamin D-binding protein	54,526	45	2	Downregulated
2010-02-07	No identification	—	—	—	Downregulated
2010-02-08	Beta-actin	42,052	210	11	Upregulated
2010-02-09	No identification	—	—	—	Downregulated
2010-02-10	No identification	—	—	—	Downregulated
2010-02-11	Pigment epithelium-derived factor	46,484	71	4	Upregulated
2010-02-12	Fibrinogen gamma chain	52,106	189	9	Upregulated
2010-02-13	Apolipoprotein A-I	30,759	215	10	Downregulated

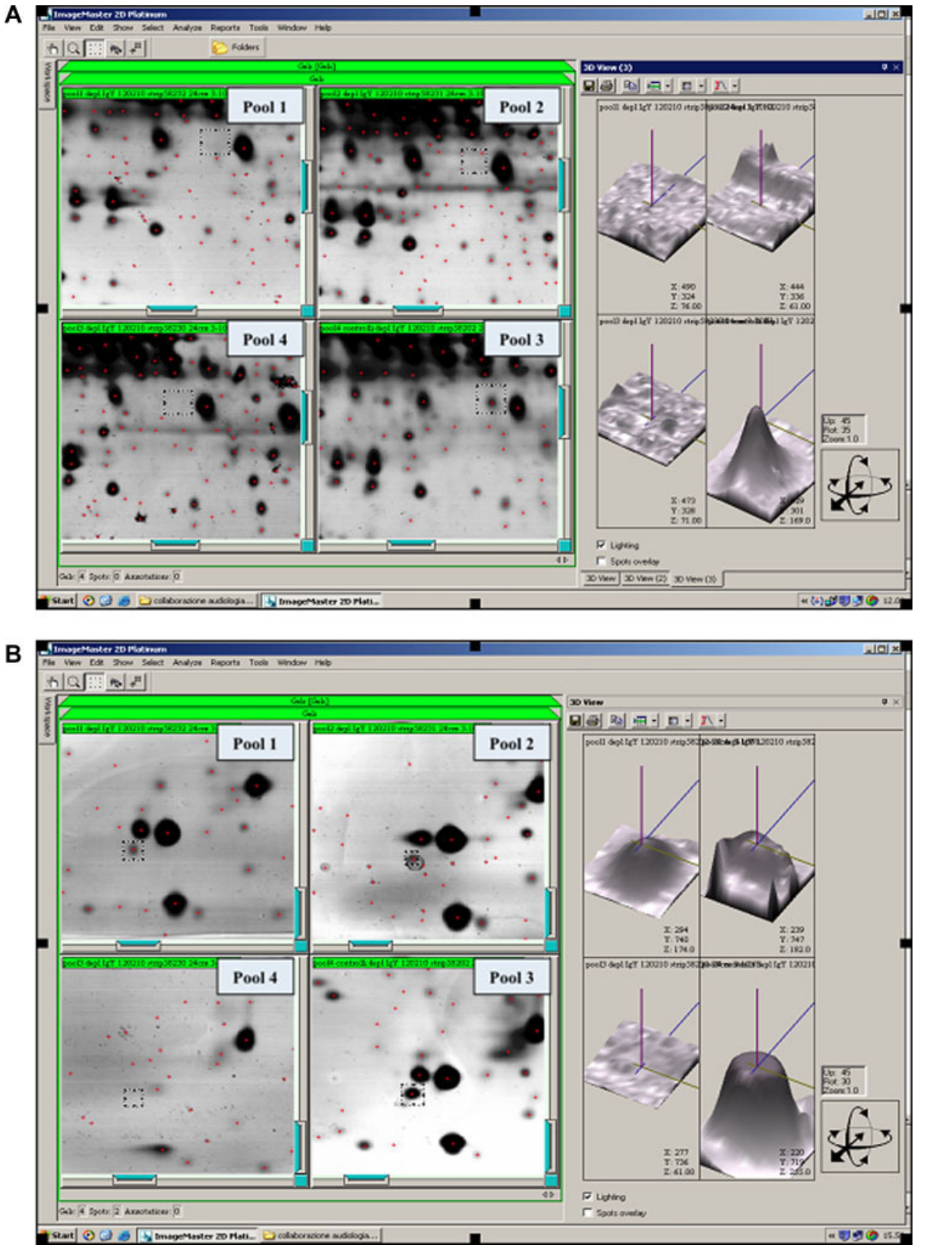

Fig. 2. Densitometric analysis of beta-2 glycoprotein (A) and apolipoprotein-I (B) performed on plasma pools. Analysis was performed using the Image Master 2D Platinum software (see Materials and Methods Section).

membrano-proliferative glomerulonephritis, atypical hemolytic uremic syndrome and age-related macular degeneration (Vogt et al., 1995; Ying et al., 1999; Li et al., 2006); CFH, and its related proteins, are strongly activated in otitis media with effusion (Närkiö-Mäkelä et al., 2001), underscoring the role played by the alternative complement pathway in the development of inflammation in this particular disease. Beta 2 glycoprotein 1 (beta-2GP1) is a 50-kDa glycoprotein that binds to negatively charged substances (heparin, dextran sulfate) preventing activation of the intrinsic blood coagulation cascade by interacting with phospholipids on the surface of damaged cells. The presence of beta-2GP1 antibodies has been recently detected in patients with idiopathic sudden sensorineural hearing loss (Toubi et al., 2004). Vitamin D binding protein (VDBP), also known as Gc-globulin (group-specific component globulin) is primarily involved in actin scavenging system, thus protecting cells from the toxic effect of intravascular actin polymerization (Lee and Galbraith, 1992). Mechanistically, VDBP has been shown to reduce platelet aggregation and to prolong coagulation time ex vivo (Gasparri et al., 2010). Beta actin is a ubiquitous protein involved in the formation of filaments that are a major component of the cytoskeleton. This protein has been identified as a potential candidate autoantigen in autoimmune inner ear disease (Boulassel et al., 2000). Moreover, beta actin mutations altering depolymerization dynamics are associated with autosomal dominant deafness, and dystonia and with non-syndromic hearing loss (Procaccio et al., 2006; Accetturo et al., 2010).

Presently, early diagnosis of MD is not an easy task, due to the low specificity of the initial symptoms; in order to rule out other problems, a detailed history and physical examination is required, supported by audio-vestibular diagnostic tests (audiometry, video-oculography, auditory evoked potentials, and vestibular evoked miogenic potentials) and imaging (magnetic resonance imaging). Our data, derived from the analysis of plasma proteome, even if not aimed at elucidating the molecular basis underlying MD development, provide a novel, not invasive and easily reproducible tool for early diagnosis and follow-up of MD patients.

The most effective relief of the symptoms in severe MD is the targeted destruction of the vestibular sensory organs, either by use of selected ototoxic drugs (gentamicin, steroids) or, if vertigo attacks associated are severe and debilitating and other treatments do not help, by surgical procedures (endolymphatic sac decompression, vestibular nerve section, labyrinthectomy).

We postulate that the protein profile obtained from plasma analysis, might help, in the near future, the development of innovative, rational and tailored therapeutic schemes for MD in a targeted, nondestructive way.

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