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a methodological journal

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JIOMICS

Journal of Integrated OMICS

Focus and Scope

Journal of Integrated OMICS, JIOMICS, provides a forum for the publication of original research papers, preliminary communications, technical notes and critical reviews in all branches of pure and applied "-omics", such as genomics, proteomics, lipidomics, metabolomics or metallomics. The manuscripts must address methodological development. Contributions are evaluated based on established guidelines, including the fundamental nature of the study, scientific novelty, and substantial improvement or advantage over existing technology or method. Original research papers on fundamental studies, and novel sensor and instrumentation development, are especially encouraged. It is expected that improvements will also be demonstrated within the context of (or with regard to) a specific biological question; ability to promote the analysis of molecular mechanisms is of particular interest. Novel or improved applications in areas such as clinical, medicinal and biological chemistry, environmental analysis, pharmacology and materials science and engineering are welcome.

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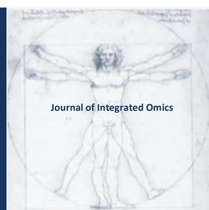

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High-throughput genomic technology in research of virulence and antimicrobial resistance in microorganisms causing nosocomial infections

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Abstract

Most hospital-acquired infections are caused by organisms common in the general population and most are relatively harmless. Infection by nosocomial pathogenic bacteria is increasingly becoming a major threat to hospital patients. Molecular diagnosis of antibiotic resistant organisms such as *Clostridium difficile* Infections (CDI) Methicillin Resistant *Staphylococcus aureus* (MRSA), Extended-Spectrum β-lactamase (ESBL) *Escherichia coli*, Vancomycin Resistant *Enterococcus* (VRE), Carbapenem-Resistant *Klebsiella* (CRK), among others, is vital for prevention of healthcare-acquired infections in acute care facilities. DNA microarray besides being a promising diagnosis tool may also provide valuable information about the mechanisms of antimicrobial resistance and pathogenicity of these bacteria. This review aims to highlight the prominence of high-throughput genomic tools in research of virulence and antimicrobial resistance in microorganisms causing nosocomial infections.

Keywords: Microarrays; Nosocomial Infections; Virulence; Antimicrobial Resistance.

1. Introduction

1.1. *Nosocomial infections*

A nosocomial infection (from the Greek word for hospital "nosokomio"), also known as a "hospital-acquired infection", is an infection acquired in hospital, or other health care facility, by a patient who was admitted for a reason other than that infection. This includes infections acquired in the hospital but appearing after discharge, and also infections among hospital staff [1].

Nosocomial infections occur worldwide and affect both developed and underdeveloped countries. Although the current progress in public health and hospital care, nosocomial infections belong to the most common infections and complications in clinical medicine and continue to develop in hospitalized patients. Infections acquired in health care settings are among the major causes of death and increased morbidity among hospitalized patients [2]. It is estimated that at any one time, about one million and half people worldwide suffer from infectious complications acquired in health care units [3]. These infections prolong hospitalization, require more extensive diagnostics and treatment, and are associated with additional costs [4, 5].

The most frequent nosocomial infections are lower respiratory tract infections, surgical wounds, urinary tract infections and sepsis. Several studies have shown that these infections most commonly occur in intensive care units and in acute surgical and orthopedic wards. Infection rates are also higher in patients with increased susceptibility such as immunocompromised, age (infants and elderly), chronic hemodialysis patients, and those receiving chemotherapy treatments [6-9].

During the stay in health care facilities, patients are exposed to a variety of microorganisms. The contact between patients and the microorganism itself does not necessarily

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result in the development of clinical disease. There are other factors which influence nosocomial infections, such as the characteristics of microorganisms, including the virulence and resistance to antimicrobial agents [10].

Different bacteria may cause nosocomial infections. Infections may be caused by the transference of a microorganism from one patient to another person in the hospital (cross-infection) or may be caused by the patient own flora (endogenous infection). In addition, infection can be spread by environmental transfer, as from an inanimate object, through inhalation of aerosols, or from a substance recently contaminated by another human source.

Most nosocomial infections are caused by organisms common in the general population, in which are relatively harmless. They may not cause disease or a milder form of disease than in hospitalized patients. This group includes the anaerobic bacteria *Clostridium difficile*, facultative anaerobic such as *Staphylococcus aureus*, coagulase-negative staphylococci, *Escherichia coli*, *Enterococcus* spp, *Klebesiella* spp, and *Pseudomonas aeruginosa*, and the obligate aerobic *Acinetobacter baumannii* [11, 12].

Furthermore, the hospital environment supports the acquisition of resistance to antimicrobial agents by bacteria, complicating the treatment of infections due to drug-resistant pathogens. Antimicrobial-resistant bacteria are of particular importance, since impair or prevent the successful treatment of infections [13]. The increasing number of antimicrobial agent-resistant pathogens and high-risk patients in hospitals are challenges to progress in preventing and controlling these infections.

2. Antimicrobial resistance and virulence

The increasing resistance to antimicrobial agents has been a global problem in recent years in both developed and developing countries and it has rapidly become a leading public health concern. Shortly after the introduction of penicillin into general medical use in the 1940s, it was recognized that bacteria would develop resistance to antibacterial agents. By 1948, most of the *S. aureus* isolated in British hospitals were resistant to penicillin due to production of β-lactamases. [14].

As other antimicrobial agents were introduced, organisms resistant to them were isolated from infected patients or from the environment. This has developed into a cycle of antimicrobial agent development, introduction into clinical use, and the development of resistance-often to the point where these drugs becomes worthless in a few years [15].

The prevalence of antimicrobial resistance varies greatly between and within countries and between different pathogens. However, the fact that many patients receive antibiotics, even when its use is not indicated, can be the main reason for the emergence of multi-resistant strains, through the selection pressure and exchange of genetic resistance elements involved in resistance. While, microorganisms in the normal human flora sensitive to a particular antimicrobial agent are suppressed, the resistant strains persist and may become endemic in the hospital. The widespread use of antimicrobial agents not only for therapeutic purposes but also for prophylactic use has been identified as the main determinant for the emergence and spread of resistant microorganisms [16].

Antimicrobial resistance bacteria are a major cause of nosocomial infections and are associated with increasing rates of mortality among hospitalised patients. Many strains of staphylococci, *Enterobacteriaceae*, and enterococci are currently resistant to most or all antimicrobials which were once effective [17, 18]. Moreover, multi-resistant *Klebsiella* spp, *P. aeruginosa*, *A. baumannii*, and *C. difficile* are prevalent in many hospitals [17, 19-23]. These pathogens are able to evade the effects of antibiotics through a significant number of mechanisms, compromising the effective treatment of infections. Health care units environment provides a selective antimicrobial pressure and a proper area for dissemination of resistance genes. The widespread use of antimicrobial drugs and the presence of sensitive microorganisms allow the acquisition and transfer of resistance genes and thus the emergence of highly pathogenic bacteria. This problem is particularly critical in developing countries where more expensive second-line antibiotics may not be available or affordable [24].

3. High-throughput genomic technology

High throughput genome-wide array based techniques such as Comparative Genomic Hybridization (CGH) and transcriptional profiling provide an opportunity to discover genes and/or pathways that are specifically activated in the different stages of bacterial infection or in response to antimicrobial drug exposure. In addition to helping to understand carriage and disease processes by pathogenic bacteria, such data provided by these tools may also contribute to antimicrobial and vaccine development through the identification of targets found in these organisms [25, 26].

3.1. *DNA microarrays*

In the era of "whole genome studies", the advent of genome sequencing and DNA microarray technology are increasingly gaining importance, as a high throughput technology to integrate gene information with biological function [26, 27].

The microarray is the most powerful genomics approach available for high through-put analysis. This tool is used to analyse both expression levels of genes in a given organism, and comparative hybridization of differentially labelled DNA from two samples. A DNA microarray is typically a glass slide on to which DNA molecules are immobilized in a random manner in specific locations called spots. A microarray may contain thousands of spots and each spot contain DNA probes that uniquely correspond to a gene. The DNA probes in a spot may either be a PCR product or

short stretch of oligonucleotide strands that correspond to a particular gene. The probes are printed on to the glass slide by a robot or using a DNA photolithography process (Affymetrix GeneChips) [28, 29]. On the other hand, the recent microfluidic hybridization method, combining the fields of microfluidics and DNA microarrays, shows several advantages such as less sample usage and reduced incubation time. These capabilities allow a rapidly and accurately detection of infectious pathogens and drug resistance markers [30, 31].

DNA Microarrays may be used to analyse gene expression in many ways, but one of the most common applications is to compare expression of a set of genes from a particular condition of bacterial cells (for example, a stress condition) to the same set of genes from a reference under normal conditions. This technology allows to analyse RNA preparations that were extracted from *in vitro*-cultivated as well as to determine the transcriptional status *in vivo*-derived bacteria at the level of the whole genome bacteria [32].

On the other hand, microarray CGH provides an estimate of the relative abundance of genomic DNA taken from test and reference organisms by hybridisation to a microarray containing probes that represent sequences from the reference organism [25]. Bacterial genome evolution is dominated by gene insertions/deletions and gene divergence [33, 34]. Genetic diversity of intra-species must be analysed if we are to gain a better understanding of the evolution of the genome of a given bacterial organism and use that information for instance for development of technical applications as vaccines or bacterial drug development [35]. [25, 35]. This will especially be valuable when particular virulence and antimicrobial resistance-associated genes are identified [25, 36-38]. Different studies on bacterial microarray CGH have demonstrated the power of the method in a comparative genomics context [34, 39].

A range of important bacterial pathogens, including multi-drug resistant isolates have been sequenced. These datasets have provided the opportunity to develop DNA microarray chips for comparative and gene expression studies, allowing the detection of antibiotic resistance and virulence genes. These microarrays are able to detect hundreds of resistance and virulence genes, and can be used to analyse a variety of diverse bacteria species including important pathogenic nosocomial organisms [40-42].

4. *Clostridium difficile*

The Gram-positive bacillus *C. difficile* is an obligate anaerobic and is the most frequent and clinically important cause of diarrhoea that has been strongly associated with the hospital setting. *C. difficile* differs from other nosocomial pathogens, since the emergence and prevalence of resistant strains is not directly linked to the treatment of the infection caused by this bacterium. The *C. difficile* infection (CDI) is nearly exclusively caused by antibiotic exposure in the treatment of other bacterial infections that disrupting the normal intestinal flora, allowing *C. difficile* to flourish. Many consider antimicrobial agents usage and poor hygiene within a clinical setting as key underlying factors of CDI outbreaks [43].

In the last decade the incidence of CDI has increased and significant outbreaks in several hospitals have been associated with a high number of cases of toxic megacolon, colectomy and mortality [44]. A recent review of mortality due to CDI, found attributable mortality of 8.03% in studies performed since the year 2000, compared with 3.64% in those before this date [45]. *C. difficile* is also associated with increased health care costs. CDI represents a significant problem to healthcare resources as treatment regimen requires spatial isolation of patients, intensified measures for infection control and the frequent use of disinfectants on surfaces and medical equipment. However, one of the main reasons by which *C. difficile* increases costs is by extending the length of time patients spend in hospital. Patients with CDI spend 7–21 days longer in the hospital and cost at least 50 % more, compared to patients who do not develop CDI during hospitalization [46].

The emergence of CDI is believed to be associated with ageing population, and due to the dissemination of hypervirulent clones. In particular, the mutant hypervirulent strain PCR ribotype 027 (North American) has been found to produce 16-fold more toxin A and 23-fold more toxin B in addition to the binary toxin. This global epidemic strain has also been reported to cause outbreaks in Europe with increased morbidity and mortality [47-49].

Resistance to erythromycin, clindamycin and fluoroquinolones is exhibited among most *C. difficile* pathogenic strains. Ribosomal methylation mediated by *erm* genes confers high level resistance to erythromycin and clindamycin. In addition, two main mechanisms of fluoroquinolone resistance have been identified, such as amino acid substitutions in the quinolone-resistant determining region of target enzymes, widely spread in many bacteria; and decreased antibiotic accumulation inside the bacterium due to an overexpression of efflux pump systems [50]. Vancomycin and metronidazole are the antibiotics of choice to treat CDI, although vancomycin has been shown to be more effective in patients with severe CDI. However, CDI is an ongoing challenge since about 20% of the treatments with metronidazole or vancomycin fail [51, 52].

Fidaxomicin is a first-in-class macrocyclic antibiotic being developed as a therapy for CDI and presents advantages over other antimicrobial agents used to treat CD. Fidaxomicin is clearly more potent *in vitro* than vancomycin against clinical isolates of *C. difficile*. In addition, this compound is minimally absorbed after oral administration, achieves high concentrations in the intestinal tract, long post-antibiotic effect, and restricted activity against normal gut flora, providing active and selective therapy for CDI, has little activity for inhibiting other bowel flora species [53, 54].

Several techniques are used to understand the

epidemiology and pathogenicity of *C. difficile* strains. Microarray CGH comparison studies between *C. difficile* strains revealed that only 16-19.7% genes were shared by all strains [55, 56]. The common core gene set containing conserved genes in all tested *C. difficile* strains is unusually lower than the core genome estimates of other bacterial species [57]. One contributing cause to the emergence of the PCR ribotype 027 strain is its increased resistance to antibiotics, including fluoroquinolones. A Microarray CGH study across *C. difficile* strains, from different origins, showed different levels of divergence of coding sequences involved in antibiotic resistance [55].

Under conditions that are not favourable for growth, *C. difficile* produces metabolically dormant endospores via asymmetric cell division. The use of Genome-wide microarray approach in *C. difficile* 630, an epidemic, virulent and multi-drug-resistant strain, showed a highly dynamic gene expression during germination and outgrowth [58].

CodY, a global regulatory protein that monitors the nutrient sufficiency of the environment has shown to be a potent repressor of toxin gene expression in *C. Difficile*. DNA microarray analysis, using a *codY* null mutation strain showed a overexpression of 146 genes, including metabolic and a major group of virulence genes. The coregulation of these genes by CodY underscores the connection between nutritional sufficiency and pathogenesis in this bacterium [59].

5. *Staphylococcus aureus*

Most of the medical literature shows that *S. aureus*, is certainly the most notorious and prevalent Gram-positive nosocomial pathogen found in clinical samples, being a leading cause of both skin structure infections and blood stream infections with considerable morbidity and mortality [60-62].

The seriousness of the infections caused by *S. aureus* is linked to the different potential infected tissues, ranging from skin and soft tissues to lower respiratory tracts and bloodstream. Moreover, these infections are intensified by the bacterial potential to develop multiple antimicrobial resistances. The most striking example is those conferring resistance to methicillin and other β-lactam antibiotics, known as methicillin-resistant *S. aureus* (MRSA). In many countries, most hospital associated *S. aureus* strains are resistant to methicillin, with MRSA rates upper 50% [63]. However, there are considerable geographical variations in MRSA rates when infections are grouped by continent of origin. MRSA rates are especially high in North America and Asia, and are significantly lower in western Europe [61].

Hospitalized patients whose immune systems are weak show a high frequency of *S. aureus* infections. Besides, infections by *S. aureus* in these kind of patients may develop into extremely dangerous and life-threatening diseases such as osteomyelitis, meningitis, necrotizing pneumonia and infective endocarditis [61, 64].

The antimicrobial agent vancomycin has been first-line antibiotic treatment for serious infections caused by MRSA, including complicated skin-structure infections, bloodstream infection, and pneumonia [64]. However, despite being the criterion standard therapy, the susceptibility of MRSA to this antimicrobial agent may be decreasing, and reports of clinical failure are increasing [65, 66]. Moreover, antimicrobial drug requires intravenous administration, and occasionally patients experience unacceptable side effects. Linezolid, a member of the new oxazolidone class of antibiotics, is highly active *in vitro* against MRSA and has excellent oral bioavailability. However, the emergence of linezolid -resistant *S. aureus* has been reported in recent studies [67, 68]. The emerging potential of vancomycin and linezolid resistant *S. aureus*, provides a serious concern for the future treatment of hospital-acquired infections.

An atypical MRSA strain was isolated during an epidemiological survey of *S. aureus* in cystic fibrosis patients, in France. Genome analysis of this MRSA isolate using high throughput sequencing method and microarray CGH analyses revealed the presence of a new antibiotic inducible phage [69]. Antibiotic-mediated phage induction may result in high-frequency transfer and the unintended consequence of promoting the spread of bacterial virulence and/or antibiotic resistance determinants. Expression microarrays, showed that the genes differentially expressed between strains from cystic fibrosis patient and non-cystic fibrosis patient, involve phage elements or resistance determinants [69]. In addition, CGH comparisons between hospital- and community-associated isolates in Canada have revealed genetic differences which included open reading frame encoding potential virulence factors [39]. Moreover, in Romania, one of the countries with the highest prevalence of MRSA, several *S. aureus* clinical isolates from different infections were recently characterized by microarray hybridisation [70].

Rhodomyrtone, is a natural antibacterial drug displayed significant antibacterial activities against MRSA. Microarray analysis showed a significant modulation of gene expression, in MRSA exposed to subinhibitory concentrations of rhodomyrtone. Genes up-regulated included genes involved in metabolism of amino acids, which can be related with the antimicrobial mechanism of rhodomyrtone. In addition, overexpression of virulence factors was also detected [71]. Moreover, a recent microarray analysis was used to investigate changes in gene expression in the EMRSA-15 strain (NCTC 13142), exposed to manuka honey, a broad -spectrum antimicrobial agent. In this analysis, the microarray data indicated notable changes in expression of several genes, with potential clinical significance, including important MRSA virulence determinants [72].

6. Coagulase-negative staphylococci

Coagulase-negative staphylococci (CoNS) are a heterogeneous group of opportunistic pathogens whose adaptability to persist and multiply in a variety of environments causes a wide spectrum of diseases in humans. Species such as *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* are commonly found on the skin and mucous membranes of humans and are therefore often found in clinical specimens [73]. *S. epidermidis* is the most frequently isolated member of the group of CoNS has emerged as a major cause of nosocomial infections. It has been associated with bacteremia, wound-related infections, postoperative infections, and most important intravascular catheter–related infections.

Usually an innocuous commensal microorganism on human skin, CoNS can cause severe infection after penetration of epidermal and mucosal barriers, which frequently occurs in patients during the insertion of indwelling medical devices [74]. In these patients, the host defence mechanisms often seem unable to handle the infection and, in particular, to eliminate the staphylococci from the infected device because of the development of a biofilm on the foreign body surface [73, 75, 76].

S. epidermidis differs from *S. aureus*, by its inability to produce coagulase and usually not cause pyogenic infections in non-compromised patients, with the exception of native valve endocarditis [77]. Because of the lack of severely toxins tissue-damaging exoenzymes, compared to *S. aureus*, *S. epidermidis* infections are usually subacute or chronic. [74]. The success of *S. epidermidis* infection, is usually attributed to its ability to adhere to surfaces and to remain there, being the biofilm formation considered to be the main virulence factor [78].

Treatment of CoNS infections is generally difficult due to the ability of these bacteria to develop resistance to all described antibiotics, and because the slime capsule of staphylococci represents an almost impermeable barrier to many antibiotics [79]. Moreover, methicillin-resistant CoNS (MRCoNS) are currently a common finding among hospitalized patients [80, 81]. Therefore, glycopeptide antibiotics (vancomycin and teicoplanin) are usually the antimicrobial drug of choice for treatment of infections by MRCoNS [82, 83]. However, in the last decade decreased susceptibility of CoNS isolates to glycopeptides has been reported from different parts of the world, and thus representing an emergent challenge to the clinicians [84-86].

Comparative genomics has been used as an alternative approach to identify bacteria virulence determinants. A study performed between clinical and benign *S. epidermidis* strains revealed a high genetic variability of this bacterium as a species. Several markers were identified for *S. epidermidis* invasiveness, which included proposed virulence factors and potential targets for drug development against *S. epidermidis* infections [87].

Biofilm formation leads to a non-aggressive and protected form of bacterial growth with low metabolic activity, which is optimally suited to guarantee long-term survival during chronic infection. The development of a biofilm is accomplished through a series of sequential steps, each of which is characterized by changes in gene expression in response to environmental signals and cell-cell signalling [88]. The gene expression profile of a *S. epidermidis* during biofilm development was analysed by [89] using a DNA microarray representing its entire transcriptome. In this study, the pattern of gene expression in *S. epidermidis* biofilms is characterized by a distinct physiological state which presumably results in increased protection from antibiotics and the immune defense of the host and allows bacteria to persist during infection.

Moreover, a microarray developed to detect of up to 90 antibiotic resistance genes in Gram-positive bacteria, was recently used to identified antibiotic resistance genes in MRCoNS strains isolated from pets and horses [90].

7. *Escherichia coli*

E. coli is the head of the large bacterial family, *Enterobacteriaceae*, the enteric bacteria, which are facultatively anaerobic Gram-negative, and is commonly found in the intestinal tract of humans. The emergence of multi-resistant *E. coli* has been observed, identifying it as a major threat to public health. European studies, showed that *E. coli* exhibited a Europe-wide increase of resistance to fluoroquinolones, third generation cephalosporins and aminoglycosides [91].

The emergence and wide dissemination of extended spectrum β-lactamases (ESBLs) among clinical *E. coli* isolates in hospitals, has caused a major concern in several countries, being frequently implicated in human infections. These infections have a great impact on public health due to an increased incidence of treatment failure and severity of disease. ESBLs mainly include TEM, SHV, and CTX-M enzymes. Among them, the highest number of variants described during the last years corresponds to the CTX-M family [92]. The presence of CTX-M enzymes render *E. coli* resistant to a variety of β-lactams, and are transferred via plasmids that can also include resistance genes to several unrelated classes of antimicrobial agents [93].

The dissemination of CTX-M enzymes around the world has been referred as the “CTX-M pandemic” mostly because of the increase of reports worldwide. One of the most interesting issues in the dispersion of CTX-M enzymes from *E. coli* is the participation of specific clones. New approaches based on MLST typing have demonstrated that despite a high diversity among CTX-M producers, a few clones or sequence types grouped in clonal complexes have been repeatedly found linked to CTX-M enzymes. This suggests that they are involved in the dissemination of these enzymes and that the adaptive success of some CTX-M enzymes could also depend on specific sequence types or clonal complexes where they are frequently present [94].

Although *E. coli* is not traditionally associated with

nosocomial infections, due to the acquisition of resistance determinants, it has emerged as the leading Gram-negative pathogen responsible for bloodstream and urinary tract infections [95, 96]. The invasive *E. coli* infections are mainly due to extraintestinal pathogenic *E. coli* (ExPEC) which often originate from the urinary tract (uropathogenic *E. coli*, UPEC) [97]. ExPEC strains are genetically distinct from commensal *E. coli* found in the intestinal flora. They are usually characterized by a predominance of phylogenetic group B2, and encode a large number of virulence factors responsible for pathogenesis outside of the gastrointestinal tract. The virulence factors belong to various functional groups among which adhesins, toxins, iron sequestration systems, and polysaccharide coatings. These virulence factors are necessary for bacteria to overcome innate host defences, invade host tissues, and to trigger a local inflammatory response [98].

Several studies using DNA microarrays have been conducted in *E. coli* isolates to detect antimicrobial resistance genes and virulence genes [99-102]. In studies performed in *E. coli* strains from different sources, CGH approach has shown to be a valuable tool for understanding the clonality of pathogenic *E. coli*, by defining the core genome, identifying regions of variation, and identifying antimicrobial and virulence-associated genes [34, 38, 103, 104].

cDNA microarray screening for the gene expression analysis in fluoroquinolone-resistant and sensitive *E. coli* were performed recently in clinical isolates from UTI patients. The upregulation of phage shock protein operons, *pspC* and deoxyribonucleic acid adenine methyltransferase were suggested to contribute to acquiring fluoroquinolone resistance [105].

8. *Enterococcus spp*

Enterococci are Gram-positive bacteria that typically colonise the gastrointestinal tract of humans and animals, and may also colonize the upper respiratory tract, biliary tracts and vagina of otherwise healthy persons [106, 107]. Most enterococci are not virulent and are considered relatively harmless, with little potential for human infection. However, they have also been identified as nosocomial opportunistic pathogens with increased resistance to antimicrobial approved agents causing infections, most commonly, urinary tract infection, but also cholecystitis, cholangitis, peritonitis, septicemia, endocarditis, meningitis, and surgical site infections in hospitalised patients [108, 109].

The genus Enterococcus includes more than 17 species, but only two species, *Enterococcus faecalis* and *Enterococcus faecium*, account for most clinical infections in humans. Other enterococcus species, *E. gallinarum*, *E. casseliflavus*, *E durans*, *E. avium*, *E. hirae*, *E. malodoratus*, *E. mundtii*, *E. raffinosus*, and *E. solitarius* are isolated much less frequently and represent less than 5% of clinical isolates. However, frequent misidentification by classical biochemical or microbiological methods suggests that their importance might be underestimated [110-113]. Accurate species identification of enterococci has become important, in particular because some species have been recognized as human pathogens following the wide prevalence of acquired antibiotic resistance [114].

Enterococci are intrinsically resistant to many antimicrobials and have the ability to acquire resistance to glycopeptides and aminoglycosides, among others, posing a challenge to therapeutic options [115]. Multidrug-resistant enterococci have been increasingly identified as the most important pathogens responsible for nosocomial infections in man [109]. The acquisition of vancomycin resistance by enterococci (VRE) has seriously affected the treatment and infection control of these organisms. VRE, particularly *E. faecium* strains, are frequently resistant to most antimicrobial agents that are effective in the treatment of enterococcal infections, which leaves clinicians with limited therapeutic options. VRE colonization and infection occurs predominantly in patients with severe underlying illness, extended length of hospital stay, and previous antibiotic exposure. The development of newer antimicrobial drugs, such as quinupristin-dalfopristin, linezolid, daptomycin, and tigecycline with activity against many VRE strains has improved this situation; however resistance to these agents has already been described [116].

Clonal spread is the dominant factor in the dissemination of multidrug-resistant enterococci in North America and Europe [117]. Virulence and pathogenicity factors have been described using molecular techniques. Several genes isolated from resistant enterococci encoding for the virulence factors such as aggregation substance, surface adhesins, sex pheromones, lipoteichoic acid, extracellular superoxide, gelatinase, hyaluronidase, and cytolysin (hemolysin). Each of them may be associated with various stages of an endodontic infection as well as with periapical inflammation [118].

Enterococci species show significant differences in the incidence of virulence factors. Generally, *E. faecalis* appears to harbour more virulence determinants than *E. faecium* strains which are generally free of virulence factors [119]. On the other hand, considering the distribution of the antibiotic resistance according to the species, the *E. faecium* shows a higher level of resistance than *E. faecalis* [120, 121].

Due to the versatile nature of enterococci as a commensal and as a pathogen and its impact on human health, it is of great interest to study the mechanisms that are related to the pathogenic condition. Considerable information can be obtained by studies of the genetic diversity of the species. The use of microarrays can allow the analysis of genomic diversity in detail, obtaining indications regarding the evolution of the strains within a species.

CGH Microarrays compared *E. faecalis* from different sources against the hospital V583 strain showed considerable diversity in gene content. The percentage of divergent genes in these test strains varied from 15% to 23%, and the main variation was found in regions corresponding to

exogenously acquired or mobile DNA in V583. Virulence factors, antibiotic resistance genes, and integrated plasmid genes dominated among the divergent genes [36].

Oxidative stress works as an important host/ environmental signal that generate a wide range of responses in bacteria. A recent transcriptome study in *E. faecium*, using DNA microarray showed that the AsrR regulon (antibiotic and stress response regulator) was composed by 181 genes, including diverse groups involved in pathogenesis, antibiotic and antimicrobial peptide resistance [122]. DNA microarrays also provides an opportunity to combine the principles of transposon mutagenesis and microarray-based screening technology to identify potentially important bacterial virulence or resistance genes. A microarray-based transposon mapping was developed by [123] to identify *E. faecium* genes that contribute to ampicillin resistance. In this study, several novel mechanisms that contribute to ampicillin resistance in *E. faecium* were identified.

Rapid organism identification via molecular diagnostic assays can help to decrease the time to appropriate antimicrobial therapy. The Verigene Gram-Positive Blood Culture (BC-GP) Test utilizes microarray technology to detect specific bacterial DNA from positive patient blood cultures. This approach allows identifying genus, species, and genetic resistance determinants for a broad panel of Gram-positive bacteria directly from positive blood culture bottles. This microarray technology seems to be useful in optimizing antimicrobial therapy in bloodstream infections caused by *Enterococcus* species [124].

9. *Klebsiella spp*

Klebsiella species are important pathogens, responsible for causing a spectrum of nosocomial-acquired infections, particularly in intensive care units. *Klebsiella* spp, as opportunistic pathogens mostly cause infections in immunocompromised patients who are hospitalized and suffer from severe diseases such as diabetes mellitus or chronic pulmonary obstruction. Nosocomial *Klebsiella* infections are caused mainly by *Klebsiella pneumoniae*, clinically the most important species of the genus. *Klebsiella oxytoca* also has been isolated from clinical specimens of patients, however to a much lesser extent [125]. It has been estimated that *Klebsiella* spp is responsible for 3 to 8% of all nosocomial bacterial infections, with urinary tract infection, pneumonia, and primary bacteremia being the most common manifestations. Strains of *K. pneumoniae* are the second most common cause of Gram-negative bacteremia [126]. *Klebsiella* spp can survive and multiply in nutritionally poor, humid environments at room temperature. Subsequently, *Klebsiella* species may contaminate food, enteral feedings, and infusion fluids, leading to common-source outbreaks [127].

Klebsiella species show extensive antimicrobial agents resistance profiles, including third generation cephalosporins, aminoglycosides and quinolones. This is especially true for ESBL-producing *Klebsiella* spp. The emergence of *K. pneumoniae* producing ESBL has been reported as an important cause of nosocomial infection in the Europe and United States. The prevalence of ESBL-producing *K. pneumoniae* strains in hospital environment ranges from 5 to 25% in several places of the world [128]. Since ESBL production frequently is accompanied by multi-resistance to antimicrobial agents, therapeutic options become limited.

Carbapenems such as imipenem or meropenem possess the most consistent activity against ESBL-producing *Klebsiella* strains. Both antibiotics are considered the agents of choice in the treatment of infections due to ESBL-producing organisms [129]. However, *Klebsiella*-producing carbapenemases have rapidly emerged and disseminated worldwide. The carbapenemases hydrolyze all β-lactam antibiotics, including carbapenems, and their high potential for rapid, wide dissemination constitutes a major clinical and public health threat [129, 130].

Although several virulence factors are described as being involved in the infective potency of the community-acquired strains, data concerning for virulence determinants expressed by nosocomial strains of *Klebsiella* species are scarce. However, most clinical isolates possess a well-defined polysaccharide capsule that appears to be a critical virulence factor [131]. The capsular material forms thick bundles of fibrillous structures covering the bacterial surface in massive layers, preventing the bacterium from phagocytosis by polymorphonuclear leukocytes [132].

Significant genomic diversity, using genomic shotgun array was shown among *K. pneumonia* pathogenic isolates obtained from nosocomial infections and community-acquired in Taiwan hospitals. This genomic microarray was conducted using probes from NTUH-K2044 genome, a strain isolate from a patient with liver abscess and meningitis. Hierarchical cluster analysis of these clinical isolates showed three major groups of genomic insertion-deletion patterns that correlate with the strains clinical features, antimicrobial susceptibilities, and virulence phenotypes with mice [133]. Moreover, [134] examined the genetic diversity among *K. pneumoniae* clinical isolates, using a genomic microarray containing probe sequences from multiple *K. pneumoniae* strains. That study has shown that a genomic region containing the citrate fermentation genes was not universally present in all strains. This region contains genes that contribute to the adaptation of bacteria to different nutritional conditions.

The DNA microarray is also commonly used in transcriptome analysis. A study to evaluate differential gene expression was performed amongst susceptible *K. pneumoniae* isolate and a resistant clinical derivative. A different pattern of gene expression profile was observed in resistant isolate when compared with the susceptible isolate [135]. Also, microarray analyses were performed to determine the RarA regulon

RarA is a newly identified AraC-type regulator that is associated with the multidrug resistance phenotype of

various unrelated classes of antibiotics (chloramphenicol, ciprofloxacin, norfloxacin, olaquindox, tetracycline, and tigecycline). Transcriptome analysis, using bespoke microarray slides demonstrated the role of RarA in the MDR phenotype of *K. pneumoniae* [136].

10. *Pseudomonas aeruginosa*

Although much of the medical and scientific attention has been focused on Gram-positive multidrug-resistant bacteria, such as MRSA and VRE, resistance within Gram-negative bacilli continues to increase, creating situations in which few or no antibiotics that retain activity are available.

P. aeruginosa is a Gram-negative bacterial opportunistic pathogen that is able to cause a wide range of invasive diseases and nosocomial outbreaks. It is a common pathogen in hospitals and particularly in intensive care units, affecting mainly critically ill and immunocompromised patients [137, 138]. The infections caused by *P. aeruginosa* have been considered to be polyclonal endemic infections that follow secondary endogenous intestinal and primary respiratory tract colonization in patients who have previously received antimicrobial drug therapy [139]. This bacterium is the most common Gram-negative organism associated with nosocomial pneumonia [140], and has the potential to become extremely harmful especially for cystic fibrosis patients, who are easily affected by chronic lung infections.

Patients infected by this species are more likely to develop multiple organ failure and to die than patients with other types of pneumonia. Nevertheless, outbreaks caused by some particularly multi-resistance strains have also been reported. Usually these outbreaks are normally circumscribed in time and space and are assigned to a point source of infection which can be identified in the environment [141, 142].

P. aeruginosa has become increasingly resistant to various antimicrobial agents and frequent multi-drug resistance are associated with nosocomial strains [143]. Previous reports showed that infections in patients by *P. aeruginosa* were related to empirically treatment with inappropriate antimicrobial agents and thus to a significantly higher mortality rate [144, 145]. Broad-spectrum β-lactams such as carbapenems, are potential antimicrobial agents for the therapy of infections caused by *P. aeruginosa*. However, the emergence of carbapenem-resistant *P. aeruginosa* isolates has increased due to the intensified use of these compounds, limiting treatment options [146]. Fluoroquinolones also show potency against *P. aeruginosa* responsible for hospital-acquired infections. Ciprofloxacin, due to its potent activity against *P. aeruginosa* is most frequently quinolone for treatment of infections cause by this bacterium. Levofloxacin, a respiratory quinolone has also been widely used in recent years. However, the use of Levofloxacin was associated with an increased incidence of fluoroquinolone resistant *P. aeruginosa*, while the use of ciprofloxacin is not described as relating with this association [147].

In an infection cause by an individual *P. aeruginosa*, the virulence factors have a primary importance to establish and maintain the infection, and the expression of a particular virulence determinate depends of the infection type. *P. aeruginosa* is capable to encode an impressive range of virulence determinants responsible for pathogenesis, and are described as belonging to adhesins and other secreted toxins [148].

For epidemiological purposes, discriminating *P. aeruginosa* isolates is essential to define distribution of clones among hospital environments, and to correlate clones to their source. A genotyping study, using species-specific oligonucleotide-microarray with clinical *P. aeruginosa* strains isolated in Italian hospitals have shown that microarray typing provides a genotype definition which is particularly suitable for epidemiological studies [149].

P. aeruginosa is highly resistant to antibiotic treatment, largely due to its ability to form biofilms. Bacterial communication via quorum sensing (QS) has been reported to be essential for the creation of mature and differentiated biofilms in this organism. High-density oligonucleotide microarrays used to analyse global gene expression patterns, modulated by QS regulons in *P. aeruginosa*, have shown that several genes, including genes involved in resistance and virulence were upregulated by QS [150]. In addition, data from cDNA microarray showed that expression of 382 genes was significantly different in *P. aeruginosa* treated with C2, a novel QS inhibitor. Forty-four of these genes are involved in transcriptional regulation, including a significantly upregulation of the *qscR*, which encodes the LuxR-type receptor QscR (quorum sensing control repressor) in *P. aeruginosa* [151].

ParA and ParB proteins in *P. aeruginosa* are important for optimal growth, nucleoid segregation, cell division and motility. In a recent study, microarray analysis showed that ParA and ParB besides their role in accurate chromosome segregation may act as modulators of genes expression, including antibiotic resistance and susceptibility factors and genes involved in virulence [152].

11. *Acinetobacter baumannii*

Although the organisms previously described have been subject of much attention as causing nosocomial infections, some other bacteria, such as *A. baumannii* has, in recent years, emerged as one of the most troublesome pathogens related with hospital-acquired infections. This Gram-negative non-fermentative coccobacillus can utilize a variety of both carbon and energy sources and is able to grow in a range of temperatures and pH conditions [153]. These properties may explain the ability of *A. baumannii* strains to persist in either wet or dry conditions in a hospital environment, thereby contributing to transmission. This hardiness, associated with multidrug-resistance, contributes to the *A. baumannii* has been increasingly reported, in the 15 years as a prevalent cause of nosocomial infections in intensive care units [154, 155].

A. baumannii it has intrinsic resistance to certain antimicrobial agents and has acquired resistance to many others including carbapenems which are drugs of choice in the treatment of severe infections, leaving few therapeutic options [156]. As a consequence of this, treatment of infections attributed to *A. baumannii* is challenging, and has been shown to increase mortality and length of hospital stay. The increase in carbapenem resistance among *A. baumannii* is mediated by two groups of β-lactamases, carbapenem-hydrolysing class D beta-lactamases and class B metallo-beta-lactamases [157]. However, the most widespread β-lactamases with carbapenemase activity are carbapenem-hydrolysing oxacillinases belonging to molecular class D. These enzymes belong to three unrelated groups of clavulanic acid-resistant beta-lactamases, represented by OXA-23, OXA-24 and OXA-58, that can be either plasmid or chromosomally encoded [158].

The bacteria commonly target the most vulnerable hospitalized patients; those who are critically ill with breaches in skin integrity and airway protection. *A. baumannii* has been implicated in a wide range of severe nosocomial infections including pneumonia, bacteraemia, meningitis, urinary tract and wound infections. Nosocomial pneumonia is the most common infection caused by *A. baumannii*, and nosocomial post-neurosurgical meningitis is much less common [159]. As multidrug-resistant *A. baumannii* infection usually occurs in severely ill patients in the intensive care unit, the associated crude mortality rate is high [160].

Despite a reputation for relatively low virulence, multidrug-resistant *A. baumannii* infection represents a very high threat to patients. The cause of many outbreaks by this organism is becoming endemic in hospital environments.

Comparative genomics of multidrug resistance *A. baumannii* analysis showed that this bacterium is a diverse and genomically variable pathogen. In addition, these studies also demonstrate the usefulness of comparative genome sequencing for analysis of putative resistance mechanisms in *A. baumannii* [161, 162].

DNA microarray is a useful tool for performing gene expression studies in bacteria. Coyne and colleagues (2010), developed an oligonucleotide-based DNA microarray to evaluate expression of genes for efflux pumps in *A. baumannii* and to detect acquired antibiotic resistance determinants. In this study has also been described a new efflux pump involved in the antibiotic resistance of *A. baumannii* [163]. More recently, a DNA microarray was designed to detect 91 target sequences associated with antibiotic resistance [164].

12. Conclusion

The issue of nosocomial infections caused by antimicrobial resistant bacteria should not be underestimated. Research efforts to clarify the mechanisms of antimicrobial resistance and microbial pathogenicity have provided valuable information. However, further development in this domain is required in order to obtain a comprehensive understanding and thus allowing the development of new alternative treatments and predict more accurately the evolution of resistance.

Microarray technology is a powerful tool that can be designed for analysis of comparative genomics hybridization (presence or absence of a gene) or for transcriptomics studies (level of gene expression). Although bacterial strains may appear identical based on current typing methods such as serotyping or even multilocus sequence typing (MLST), they may present potentially important genetic and phenotypic differences. Microarray CGH based on bacterial genome-sequenced strains has shown to have a high potential to determine the overall genetic similarity between strains, and therefore be essential in providing higher-resolution typing. Moreover, the availability of gene expression profiles for different bacterial pathogens, obtained by microarray technology has proved to be an essential resource in the study of bacterial infections.

Additionally, this technology as demonstrated to be useful for performing functional genomic analysis in nosocomial bacterial infections in order to gain a global view of the multifactorial phenomenon of antimicrobial resistance and virulence and to identify novel genes involved in pathogenesis and resistance. Much of the research using DNA microarrays aimed at identifying novel therapeutic targets for the treatment of bacterial infections and has been particularly directed at new drug development and identification of specific virulence factors and regulatory pathways that are relevant to the disease process.

Diagnostic DNA microarrays have also been developed, including for nosocomial pathogenic bacteria detection. Although, from a clinical perspective, the utility of DNA microarrays either for an isolate typing or for transcriptomic analysis is uncertain, genomics studies can provide insights that may impact in clinical decisions. Moreover, whole genome analysis tools can be necessary as a diagnostic tool, because it provides more detailed information than other typing methods, and it offers additional data about the mechanisms responsible for antimicrobial resistance phenotype or the genetic machinery necessary for bacterial pathogenesis.

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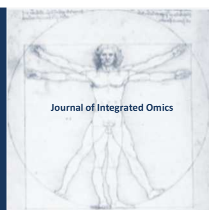

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Proteomic identification of muscle-associated biomarkers of amyotrophic lateral sclerosis using the wobbler mouse model of primary motor neuronopathy

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Abstract

Motor neuron disease is a major group of inherited or spontaneous disorders that are associated with muscular atrophy. Recently, muscle preparations from the genetic wobbler mouse model of primary motor neuronopathy have been analyzed by mass spectrometry-based proteomics. The progressive degeneration of individual motor neurons was shown to cause complex alterations in the concentration or isoform expression pattern of muscle proteins involved in the excitation-contraction-relaxation cycle, the cytoskeleton, ion handling, cellular signaling, the stress response and energy metabolism. In this article, we compare the panel of potential new muscle-associated biomarkers that have been obtained by two different, but complementary, bioanalytical approaches, i.e. label-free mass spectrometric analysis versus fluorescence two-dimensional difference-in gel electrophoresis. The complex disease-associated changes in the muscle proteome are considerably different to the more unilateral skeletal muscle transitions observed in experimentally denervated fibers or disuse-related muscular atrophy. The apparent subtype-specific vulnerability of neuromuscular synapses and compensatory mechanisms of fiber type shifting in motor neuron disease is discussed, and contrasted to other forms of muscular atrophy.

Keywords: Amyotrophic lateral sclerosis; Biomarker discovery; Motor neuron disease; Muscular atrophy; Wobbler mouse.

Abbreviations

ALS: amyotrophic lateral sclerosis; **DIGE:** difference in-gel electrophoresis; **WR**: wobbler.

1. Introduction

Mass spectrometry-based proteomics plays a central role in biomarker discovery [1]. This includes the systematic screening of both patient samples and animal models of human diseases. Ideally diagnostic biomarkers are released or leaked from pathological tissues [2] and are therefore found at a sufficient high concentration in body fluids for convenient sampling by non-invasive or minimally invasive methods [3]. However, internal tissue-associated proteins also present a crucial source for general biomarker discovery [4], including protein markers of neuromuscular disorders [5]. This important aspect of comparative tissue proteomics as related to biomarker discovery is covered in this article, which focuses on muscle-associated proteins and the proteomic discovery of novel potential markers of muscular atrophy in primary motor neuronopathy. Over the last decade, muscle proteomics has been widely applied to studying myogenesis, fibre adaptations, neuromuscular pathology and the natural aging process of contractile tissues [6-8], using both total muscle protein extracts and distinct subcellular fractions [9]. Global proteomic data supports the idea that skeletal muscle fibres are highly plastic cellular structures that can quickly adapt to changed functional demands [10]. However, if the neuromuscular system is not properly loaded due to prolonged inactivity, immobilization, lack of gravity or denervation, a relatively

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rapid process of muscular atrophy occurs [11-13]. Atrophying muscle fibres are also observed as a consequence of sarcopenia of old age and certain neuromuscular pathologies. The heterogeneous group of motor neuron diseases is a neurodegenerative disorder, which is characterized by a specific form of muscular atrophy and progressive paralysis [14]. In this review, we compare the findings from two recent studies that have analyzed the genetic wobbler (WR) mouse model of motor neuron disease by label-free mass spectrometry [15] versus fluorescence two-dimensional difference in-gel electrophoresis [16]. The systematic identification of muscular atrophy-related biomarker candidates by these two different large-scale separation and bioanalytical approaches is discussed with respect to sensitivity and coverage of proteins with differing physicochemical properties.

2. Skeletal muscle diversity

Since the highly complex isoform expression pattern of the constituents of the actomyosin apparatus are severely altered during episodes of muscular atrophy, the main regulatory and contractile proteins are briefly introduced. The biophysical and metabolic diversity of contractile tissues, as well as the physiological adaptability of whole skeletal muscles to changed functional demands, is based on an extensive variability in contractile proteins and bioenergetic enzyme isoforms [17, 18]. In human muscles, the main fiber types can be categorized as type I (slow), type IIA (fast) and type IID/X (fast), as well as a large number of hybrid fibres. Distinct mixtures of fibre types provide the contractile basis of the varying physiological demands of individual skeletal muscles [19]. The distribution of fiber type-specific myosins and metabolic enzymes is characteristic of fast-twitching fibers with glycolytic metabolism, fast-twitching fibers with an oxidative-glycolytic bioenergetics and slow-twitching fibers with predominantly oxidative metabolism. The contractile apparatus contains a variety of contractile and regulatory protein families with numerous isoforms, which have been extensively studied by proteomics [20]. Actomyosin-associated proteins can be classified by their attachment to the thick filaments located in the A-band and the thin filaments in the I-band and overlapping A-band region of sarcomeres [21]. Myosin heavy chains, which head structures are involved in the molecular coupling between myosin filaments and actin via cross-bridge/swinging-lever-arm mechanisms, represent the principal molecules of the thick filament [22-24]. Besides these main motor molecules, the regulatory and essential myosin light chains are engaged in the movement of phosphorylated myosin cross-bridges away from thick filament structures and the fine tuning of myosin motor function [25]. The actin filament-associated tropomyosin molecule plays a key inhibitory role in the regulation of actomyosin interactions [26] and the troponin complex provides the Ca^{2+}-dependent regulatory adjustment of the contractile status [27]. The troponin TnC subunit represents the central Ca^{2+}-sensor of the contractile apparatus and regulates actomyosin coupling, while the troponin TnT subunit is essential for the linkage between the inhibitory tropomyosin molecule and the troponin complex, and the troponin TnI subunit provides binding between the troponin complex and actin thereby mediating inhibition of actomyosin ATPase activity [28]. Besides myosins, actins, troponins and tropomyosins, a very large number of auxiliary proteins are present in the sarcomere structure [20], including the important class of myosin binding proteins [29]. Myosin-binding protein MBP-C is located to the thick myosin filaments and was shown to play a role in the maintenance and continuous stabilization of myosin-containing filaments [30]. Its additional function involves the modulation of cross-bridge formation between myosin and actin molecules within the complex filamentous system of contractile fibres [31], as demonstrated by electron tomography of the physical mechanism that modulates the relative sliding between thick and thin filaments [32]. Besides the isoform specific distribution of contractile proteins, the distribution of metabolic enzymes is strikingly different between predominantly slow versus fast-twitching muscles [33]. During muscle adaptations or pathological changes in the neuromuscular system, the density and/or isoform expression patterns of proteins associated with the glycolytic pathway [34] and oxidative metabolism in mitochondria [35] are majorly altered. These changes in bioenergetic enzyme profiles can be conveniently assayed by mass spectrometry-based proteomics and used to determine compensatory or disease-related shifts between anaerobic and aerobic muscle metabolism.

3. Motor neuron disease

3.1. *Motor neuron disease*

The heterogeneous group of neurodegenerative syndromes that encapsulates motor neuron diseases is associated with progressive paralysis and includes spastic paraplegia, spinobulbar muscular atrophy, hereditary spastic paralysis, primary lateral sclerosis and amyotrophic lateral sclerosis (ALS) [14]. Figure 1 gives an overview of muscle fibre type shifting due to physiological adaptations or pathological insults, including motor neuron disease and its association with muscular atrophy [36]. In general, enhanced neuromuscular activity results in fast-to-slow transitions, while neuromuscular unloading is usually characterized by slow-to-fast transformation processes. Prolonged muscle disuse, the lack of gravity, immobilization or prolonged bed rest triggers the establishment of a faster muscle phenotype. Nerve crush, experimental denervation or the effect of natural muscle aging involves progressive muscular atrophy. However, motor neuron disease was shown not to be linked to a clear slow-to-fast transformation process, but is characterized by more complex proteome-wide changes as discussed below in detail. ALS represents the most common form of motor neuron disease [37]. The adult-onset loss of lower and upper motor neurons causes a highly progressive form of paralysis, which

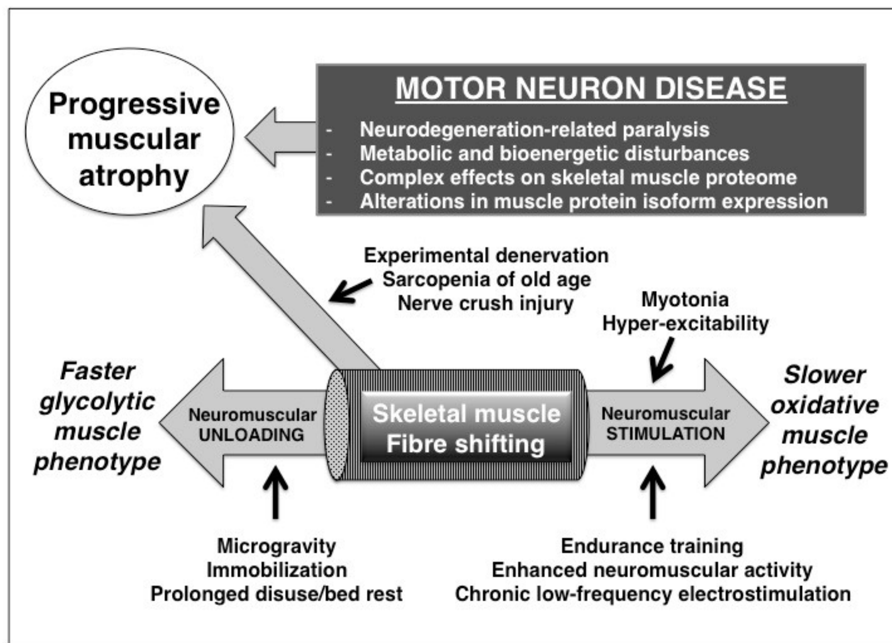

Figure 1. *Overview of skeletal muscle plasticity and fibre transitions due to physiological adaptations or pathological insults to the neuromuscular system.* Motor neuron disease is characterized by progressive muscular atrophy and complex changes in the skeletal muscle proteome.

can be sporadic or of genetic origin [38]. Neurodegeneration -related muscular atrophy results in debilitating limb and bulbar muscle weakness, as well as respiratory insufficiency [39]. A very large number of mutated genes were shown to be involved in familial or sporadic forms of ALS [40-43], including SOD1, Alsin, SETX, SPG11, FUS/TLS, VAPB, ANG, TARDBP, FIG4, OPTN, VCP, UBQLN2, SigMAR1, PFN1, ERBB4, C9orf72, CHMP2B, DAO, DCTN1, SQSTM1, hnRNPA1, Erlin2, UNC13A, NEFH, PRPH, TAF15, GRN, EWSR1 and ATXN2 [44]. Although most transcriptomic analyses of motor neuron disease have focused on spinal cord preparations and shown changed patterns of lipid metabolism, inflammation, cell adhesion and the immune response [45-47], recently alterations in early gene expression were analysed by microarray screening of the *gastrocnemius* muscle from the SOD1 mouse model of ALS [48]. Motor neuron disease appears to be a multisystem disorder with a defective muscle metabolism, which is linked to differential gene activation levels in epithelial-mesenchymal transitions and the Wnt/PI3-K signaling pathways in pre-symptomatic skeletal muscle. The inhibition of cell death, the promotion of cell proliferation and the repair of atrophying fibres seems to be impaired in the *gastrocnemius* muscle of SOD1-G93A transgenic mice. Importantly, neuromuscular impairments seem to precede motor neuron death at pre-symptomatic periods of motor neuron disease [48]. This agrees with the gene expression analysis of skeletal muscle biopsies from ALS patients that demonstrated severe alterations in mRNA levels of major muscle proteins. Drastically lower mRNA levels were described for the fast isoform of myosin binding protein MPB-C and actinin alpha-3, while the mRNAs encoding collagen, actin, myosin-8 and annexin were shown to be elevated [49].

3.2. *Wobbler mouse model of motor neuron disease*

Since comprehensive biochemical studies and subcellular fractionation procedures usually require relatively large amounts of starting material and because patient biopsy samples are scarce and show considerable inter-individual variability, genetic animal models of human diseases are often used to accumulate sufficient tissue for detailed proteomic analyses [50]. In the case of ALS research, various rodent models of motor neuron disease are used for routine pathobiochemical studies and to evaluate new pharmacological approaches [51]. One of the most established mouse models of hereditary motor neuron disease with progressive denervation is the wobbler mutant (genotype *wr/wr*, phenotype WR) [52]. A recent review by Moser et al. [53] has focused on the research progress made utilizing this animal model of ALS for studying the molecular pathogenesis of motor neuron disease. The underlying genetic abnormality in the WR mouse has been shown to be a missense mutation in the ubiquitously expressed gene *Vps54* [54]. The protein product of the affected gene is the vesicular protein sorting factor VPS54 and the mutation causes a leucine-to-glutamine replacement (L967Q) within the C-terminal domain of VPS54. The hydrophobic-to-hydrophilic amino acid exchange in the primary sequence of this crucial subunit of the hetero-trimeric Golgi-associated retrograde protein (GARP) complex results in the destabilization of its tertiary protein structure causing a reduction in the VPS54 protein

[55, 56]. Despite the fact that the WR mutation has not yet been identified in humans suffering from motor neuron disease [57], the progressive WR mouse pathology resembles many aspects of ALS [53]. The progressive neurodegenerative and neuroinflammatory processes in the WR mouse are associated with astrocyte overgrowth in the brain stem and spinal cord, hippocampal hyper-excitability, a reduced number of interneurons and muscular atrophy [58-60]. Various experimental drug treatments have been tested with the WR mouse [61-63], emphasizing the international acceptance of this ALS model mimicking the major pathophysiological and histopathological aspects of motor neuron disease [53].

4. Proteomics of muscular atrophy and motor neuron disease

Large-scale screening studies at the level of the genome, transcriptome, proteome and metabolome have been initiated to determine global changes in patient biopsy specimens and animal models of motor neuron disease [64, 65]. Besides studies on spinal muscular atrophy [66, 67], a variety of proteomic investigations have focused on ALS biomarker discovery and the pathobiochemical consequences of progressive neurodegeneration on biofluid proteomes [68, 69]. Proteome-wide profiling studies have included the comprehensive analysis of cerebrospinal fluid, cervical spinal cord specimens, lumbar spinal cord preparations and skeletal muscles [70-75]. The WR mouse model of ALS has been studied by proteomics, focusing on abnormal protein expression in skeletal muscle [15, 16] and defective spermatogenesis [76].

4.1. *Proteomic profiling of muscular atrophy*

Muscular atrophy is associated with a variety of changes in neuromuscular activity levels, such as immobilization, zero gravity, extended periods of bed rest, prolonged muscular disuse, natural aging and traumatic denervation. Skeletal muscle atrophy has a profound effect on muscle fibre type distribution with an overall tendency of a slow-to-fast transition process [13, 77]. In contrast, endurance exercise, hyper-excitability or chronic low-frequency stimulation of muscle triggers the opposite changes in fibre type specification, i.e. fast-to-slow muscle transformation, as clearly confirmed by proteomics [7, 78-80]. The fact that different loading of the neuromuscular system is reflected by distinct changes in the isoform expression pattern of contractile proteins has long been established by gel electrophoretic methodology [81] and the biochemical complexity of this process has more recently been confirmed by proteomics [20].

Proteome-wide changes during different degrees of muscular unloading were evaluated by a variety of experimental systems, including hind limb suspension, joint fixation-induced immobilization, long-term bed rest or complete denervation via sciatic nerve transection [82-96]. The general tendency of atrophying muscle to undergo slow-to-fast transitions was confirmed by changes in the abundance of a variety of metabolic enzymes, such as lactate dehydrogenase, enolase, triosephosphate isomerase and isocitrate dehydrogenase, which was accompanied by considerable alterations in contractile, structural and stress proteins [82-96]. Alterations in contractile proteins included specific isoforms of myosin heavy chains, myosin light chains, troponins and tropomyosin [89, 92, 94, 95]. Although individual studies have shown that the degree of muscular atrophy differs considerably following denervation, immobilization or prolonged disuse [85-88] and that transient alterations are initially associated with a massive decrease in contractile proteins [82-84], at a more advanced stage of fibre atrophy a metabolic oxidative-to-glycolytic shift is observed [89, 94-96]. Table 1 lists major changes in key protein families due to muscular atrophy following denervation, immobilization or prolonged disuse.

4.2. *Gel electrophoresis-based analysis of atrophying muscle in motor neuron disease*

During the early stages of establishing proteomics as a new discipline within the field of protein biochemistry, gel electrophoretic methodology has been widely employed for the efficient separating of complex protein mixtures prior to in-gel trypsinisation for the swift identification of proteins of interest [97-99]. Although the application of gel-free methods using advanced liquid chromatography coupled with tandem mass spectrometry is now extensively used in proteomic screening studies [100], fluorescence two-dimensional in-gel electrophoresis (DIGE) is still widely employed for analyzing urea-soluble muscle protein populations [101]. In muscle proteomics, a considerable proportion of proteins can be separated by routine high-resolution two-dimensional gel electrophoresis, including myosins, actins, troponins, tropomyosins and various auxiliary proteins of the contractile apparatus, as well as mitochondrial proteins, glycolytic enzymes, molecular chaperones and cytoskeletal proteins [102]. Since two-dimensional gels usually do not represent integral membrane proteins and high-molecular-mass proteins sufficiently, large one-dimensional gradient gels can be used in a complementary way to separate very large proteins prior to on-membrane digestion and subsequent mass spectrometric identification [103-105]. The flowchart in Figure 2 outlines the usage of fluorescence 2D-DIGE versus label-free LC-MS/MS analysis for the screening of pathological muscle specimens. The routine verification of novel biomarker candidates of motor neuron disease by immunoblot analysis is shown, illustrating an equal distribution of the extracellular matrix protein laminin as a loading control, the drastic decrease of the fast MBP-C isoform of myosin binding protein and increase of mitochondrial prohibitin, as previously shown by proteomics of WR muscle [15, 16].

2D-DIGE using fluorescent Cy2, Cy3 or Cy5 dyes has been originally developed by Minden et al. [106-108] and is now an established and reliable biomarker discovery method in

Proteomic study	Methods	Changed proteins	References
Analysis of total extracts from denervated rat *soleus* muscle	2D-GE, CBB, MALDI-ToF MS	Complex transient alterations during post-denervation days 1 to 10 with initial increases in AS, ENO, CA3 and fast MLC and decreases in FABP, HSP20, TnT and slow MLC	Isfort et al. [82]
Analysis of total extracts from rat *soleus* muscle following hindlimb suspension	2D-GE, CBB, MALDI-ToF MS	Transient increases of AS, ENO and CA3, as well as decreased levels of HSP20 and slow MHC	Isfort et al. [83]
Analysis of total extracts from rat *soleus* and *tibialis anterior* muscle following hindlimb immobilization by the pin-heel method	2D-GE, CBB, MALDI-ToF MS, LC-MS/MS	Complex transient alterations during post-immobilization days 1 to 10, including AS, CA3, PGM, DES, MLC, HSP60, αBC, HSP20	Isfort et al. [84]
Subproteomic analysis of cytosolic fraction from mouse *tibialis anterior* muscle following hindlimb immobilization	ICAT labeling and MS/MS analysis	Increased levels of various GLY enzymes, CA3, ICDH, HSPs and decreases in ALD, ACO, GT, MLC	Toigo et al. [85]
Analysis of total extracts from denervated rat laryngeal muscle	2D-GE, CBB, MALDI-ToF MS	Increases in CK, MLC1, MLC2, MHCIIB, TUB and PVA, as well as decreases in LDH, ENO, various OXPHOS enzymes	Li et al. [86]
Analysis of total extracts from denervated rat *gastrocnemius* muscle	2D-GE, CBB, MALDI-ToF MS	Transient changes with initial increases in ENO, αBC and HSP20, and decreases in CK, TM, ACT, MHCIIx and MHCIIB	Sun et al. [87]
Analysis of total extracts from rat *soleus* muscle following hindlimb suspension	2D-GE, CBB, MALDI-ToF MS	Muscle unloading caused a decrease in TM, ACT, MLC1, αBC and HSP20, and increases in CK, GLY and OXPHOS enzymes	Seo et al. [88]
Analysis of total extracts from rat *soleus* muscle following hindlimb suspension	2D-DIGE, MALDI-ToF MS, IB	Tail suspension triggered increases in various GLY enzymes, MYO, CA3 and ALB, and decreases in OXPHOS enzymes, ACT, TnT and a variety of MHCs and MLCs	Moriggi et al. [89]
Subproteomic analysis of rat *soleus* muscle following hindlimb immobilization	1D-GE, MALDI-ToF MS, IB	Focused study revealed preferential oxidization of CA3 and four-and-a-half LIM protein during muscle unloading	Chen et al. [90]
Analysis of proteolysis in rat *gastrocnemius* muscle following hindlimb suspension	2D-GE, CBB, MALDI-ToF/ToF MS, IB	One week of muscle unloading was associated with coordinated and time-dependent activation of proteolysis of contractile proteins; increase in GLY enzymes	Ferreira et al. [91]
Subproteomic analysis of sarcoplasmic and myofibrillar fractions from denervated rat *soleus* muscle	2D-GE, Flamingo gel stain, MALDI-ToF MS, IB	Denervation resulted in a decrease in slow MLC1 and an increase in fast MLC1; differential effects on HSPs, GLY and OXPHOS enzymes	Sato et al. [92]
Subproteomic analysis of membrane proteins from rat *soleus* muscle following hindlimb unloading	Native Blue 2D-GE, silver staining, MALDI-ToF MS, IB	Complex changes including an increase in AS and the AQP4 water channel, as well as differential effects on dystrophin-glycoprotein complex	Basco et al. [93]
Analysis of total extracts from human *vastus lateralis* and *soleus* muscle following long-term bed rest	2D-DIGE, MALDI-ToF MS, LC-MS/MS, IB	Long-term disuse caused increased MHCI, GT, various HSPs, MYO and GLY enzymes, and decreased MHCIIA, ACT and OXPHOS enzymes	Moriggi et al. [94]
Analysis of total extracts from denervated rat *tibialis anterior* muscle	iTRAQ labeling, LC-MS/MS, IB	Labeling analysis revealed large numbers of differential changes in metabolic enzymes, molecular chaperones and contractile proteins, including a decrease in many MHCs, MLCs, TM, TUB and OXPHOS enzymes, and increases in HSP70, HSP90, αBC and GT	Sun et al. [95]
Analysis of total extracts from denervated rat *tibialis anterior* muscle	iTRAQ labeling, LC-MS/MS, IB	Switch of α-isoform and β-isoform of ENO during slow-to-fast transitions, as well as differential changes of OXPHOS enzymes	Sun et al. [96]

Table 1. *Proteomic profiling of muscular atrophy*. Listed are major studies that have used mass spectrometry-based proteomics to investigate muscular atrophy due to immobilization, denervation or prolonged disuse.

Abbreviations used: αBC, alphaB-crystallin; ACO, aconitase; ACT, actin; ALB, albumin; ALD, aldolase; AS, ATP synthase; CBB, Coomassie Brilliant Blue; CK, creatine kinase; DES, desmin; DIGE, difference in-gel electrophoresis; GE, gel electrophoresis; GLY, glycolytic enzymes; GT, Glutathione transferase; ENO, enolase; FABP, fatty acid binding protein; HSP, heat shock protein; ICDH, isocitrate dehydrogenase; iTRAQ, isobaric tags for relative and absolute quantification; LC, liquid chromatography; ICAT, isotope-coded affinity tag; LDH, lactate dehydrogenase; MALDI, matrix assisted laser desorption ionization; MHC, myosin heavy chain; MLC, myosin light chain; MS, mass spectrometry; MYO, myoglobin; OXPHOS, enzymes of oxidative phosphorylation; PGM, phophoglucomutase; PVA, parvalbumin; TnC, TnI, TnT; troponin subunits; TM, tropomyosin; ToF, time of flight; TUB, tubulin.

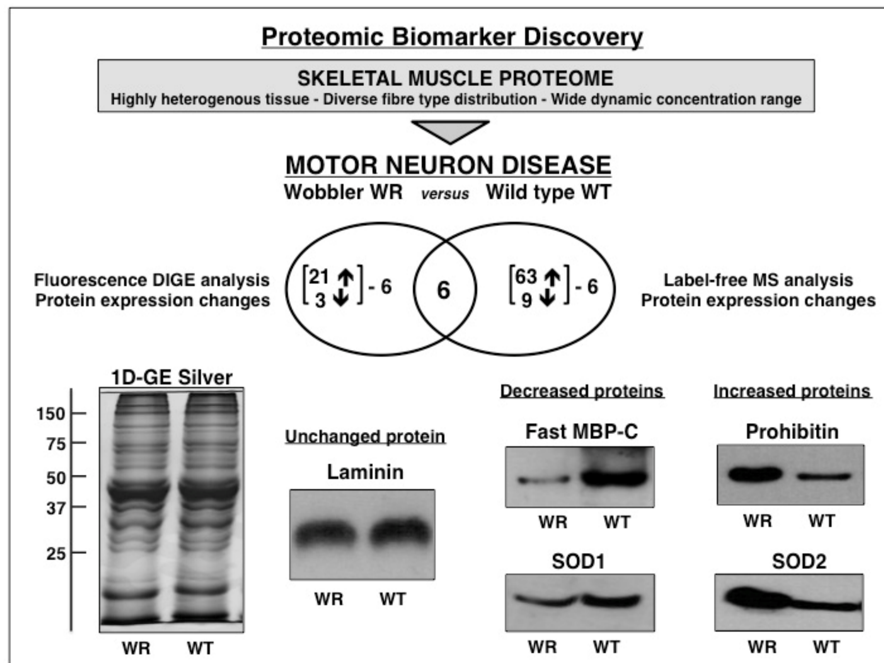

Figure 2. *Proteomic identification of novel biomarker candidates of motor neuron disease.* Contrasted are the proteomic findings of fluorescence 2D-DIGE analysis versus label-free LC-MS/MS analysis of the WR model of ALS. In the lower panel are shown a representative silver-stained gel of wobbler (WR) versus wild type (WT) muscle preparations and corresponding immunoblots labeled with antibodies to laminin, fast myosin binding protein MBP-C and prohibitin, as well as the SOD1 and SOD2 isoforms of superoxide dismutase. Gel electrophoresis and immunoblot analysis was carried out as previously described in detail [15, 16].

skeletal muscle pathology [109]. Fluorescent 2-CyDye or 3-CyDye systems are used to establish differential and pre-electrophoretic tagging of more than one proteome, thereby decisively reducing gel-to-gel variations during comparative protein separation [110]. In conjunction with optimized 2D software analysis tools [111], the advanced DIGE technique represents an unparalleled analytical tool for the establishment of distinct differences in protein expression patterns between normal and diseased tissue specimens [112]. Changes in skeletal muscles from the SOD mouse [75] and the WR mouse [16] model of motor neuron disease have been recently studied by 2D-DIGE analysis. Table 2 lists key findings from these proteomic studies of motor neuron disease and Figure 3A shows graphically the identified protein species with a differing abundance in atrophying WR muscle. Characteristic signs of denervation have previously been shown to

Proteomic study	Methods	Changed proteins	References
Analysis of total extracts from hindlimb muscle of wobbler mouse model of motor neuron disease	2D-DIGE, LC-MS/MS, IB	Disease-associated muscular atrophy with preferential effects on specific synapse types is associated with a drastic decrease in fast MBP-C, as well as increases in MLC1, ACT, TnC, GLY enzymes, MYO, CK and DES	Staunton et al. [16]
Analysis of total extracts from hindlimb muscle of SOD mouse model of motor neuron disease	2D-DIGE, MALDI-ToF MS, LC-MS/MS, IB	Disease-associated muscular atrophy with preferential effects on specific synapse types is associated with complex changes in proteins involved in metabolism, contraction and cellular stress response, such as GLY enzymes, OXPHOS enzymes, TUB, HSP90 and αBC	Capitanio et al. [75]
Analysis of total extracts from hindlimb muscle of wobbler mouse model of motor neuron disease	Label-free MS analysis, IB	Disease-associated muscular atrophy with preferential effects on specific synapse types is associated with drastic decreases in fast MBP-C, titin and SERCA1, and increases in DES, various HSPs, AS, ALB, MLC1/3, TnI, TnC and TnT	Holland et al. [15]

Table 2. *Proteomic profiling of motor neuron disease.* Listed are recent studies that have used mass spectrometry-based proteomics to investigate muscular atrophy due to motor neuron disease.

Abbreviations used: αBC, alphaB-crystallin; ACT, actin; ALB, albumin; AS, ATP synthase; CK, creatine kinase; DES, desmin; DIGE, difference in-gel electrophoresis; GLY, glycolytic enzymes; HSP, heat shock protein; LC, liquid chromatography; MBP, myosin binding protein; MLC, myosin light chain; MS, mass spectrometry; MYO, myoglobin; OXPHOS, enzymes of oxidative phosphorylation; TnC, TnI, TnT; troponin subunits; TUB, tubulin; SERCA, sarcoplasmic or endoplasmic reticulum calcium ATPase.

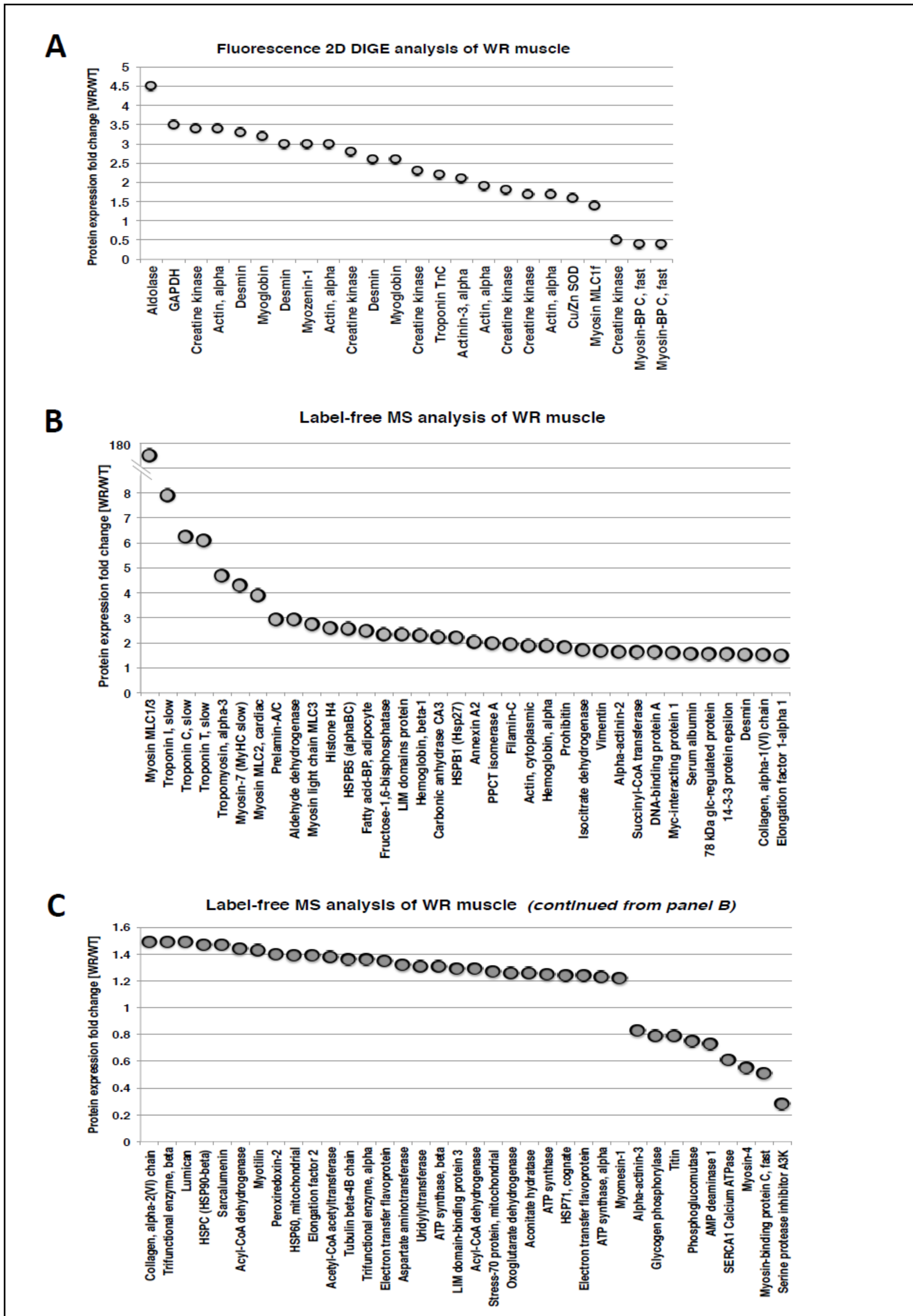

Figure 3. *Comparison of proteomic markers of motor neuron disease as determined by fluorescence difference in-gel electrophoresis (DIGE) (A) versus label-free mass spectrometric (MS) analysis (B, C) using skeletal muscle from the wobbler (WR) mouse model [15, 16].* The fold-change of individual muscle proteins is graphically presented and clearly shows that the application of label-free mass spectrometry has resulted in a larger number of identified protein species with a wider range of concentration changes as compared to gel-based proteomics.

occur in WR skeletal muscles, including an increase of mRNA levels encoding the principal α-subunit of the nicotinic acetylcholine receptor at the junctional folds [113] and a drastic shift to a high proportion of the fast-glycolytic MHCIIB isoform of myosin heavy chain [81].

However, the DIGE analysis of the WR muscle proteome has demonstrated that global changes in motor neuron disease are highly complex [16] and differ from the mostly unilateral shifts in isoform expression patterns observed following experimental denervation or prolonged muscle disuse [89, 92, 94]. Increased expression levels in WR muscle were shown for the glycolytic enzymes aldolase and glyceraldehyde 3-phosphate dehydrogenase, the contractile and cytoskeletal proteins actin, desmin, myozenin, troponin TnC, actinin and fast myosin light chain MLC1f, as well as myoglobin [16]. The M-type isoform of creatine kinase exhibited differential changes in a variety of 2D spots, which suggested the presence of differently phosphorylated isoforms following gel electrophoretic separation. An interesting finding was the elevated level of Cu/Zn superoxide dismutase, an enzyme of crucial importance for the antioxidant defence of skeletal muscle fibres, which is intrinsically involved in various forms of ALS [114]. As illustrated in the immunoblot analysis of Figure 2, the abundance of the mostly cytosolic SOD1 isoform and the mitochondrial SOD2 isoform of superoxide dismutase is differently affected in WR skeletal muscle. The 2D-DIGE analysis of the WR model also revealed the drastic reduction in the density of protein spots representing the fast isoform of myosin binding protein MBP-C [16], which has been clearly confirmed by immunoblotting (Figure 2). This makes the muscular atrophy-related modulations in MBP-C, in conjunction with concentration changes in myosin light chains, myosin heavy chains, actin, troponin, actinin and myozenin, an interesting finding and suggests MBP-C as a novel muscle-associated biomarker candidate of motor neuron disease. Interestingly, the cardiac equivalent of this regulatory protein of the contractile apparatus, cMBP-C [115], was shown to be cleaved during ischemic injury to the heart in a phosphorylation-dependent manner [116] and the release of an N-terminal fragment into the circulation may be useful as a new biomarker of diagnosing myocardial infarction [117].

4.3. *Label-free mass spectrometric analysis of atrophying muscle in motor neuron disease*

In contrast to gel-based methods, liquid chromatography in combination with advanced mass spectrometry can routinely identify low copy numbers of integral membrane proteins, high-molecular mass proteins and components with extreme isoelectric points and/or post-translational modifications. Figure 2 shows the comparison of total numbers of identified proteins using label-free MS analysis versus the DIGE method. While the fluorescence 2D-DIGE analysis identified 21 decreased protein spots and 3 decreased protein spots in WR muscle samples [16], the label-free mass spectrometric analysis of the same tissue preparations revealed 63 increased proteins and 9 decreased proteins in mutant muscle, including integral membrane proteins such as the Ca^{2+}-ATPase of the sarcoplasmic reticulum [15]. The mass spectrometric identification of the SERCA1 isoform of the luminal Ca^{2+}-pump demonstrates the advantageous and complementary nature of the gel-free LC-MS/MS method for detecting changes in a highly hydrophobic muscle protein. The considerably larger number of proteins identified by label-free mass spectrometry is graphically presented in Figure 3B,C. In analogy to the findings from 2D-DIGE studies of motor neuron disease [16, 75], label-free LC-MS/MS analysis confirmed the pathobiochemical complexity of changes in the WR muscle proteome due to progressive neurodegeneration [15]. Proteome-wide alterations included components associated with energy metabolism, metabolite transportation, muscle contraction, ion homeostasis, structural integrity and the cellular stress response [15].

A bioinformatic STRING analysis of the proteomic data from the label-free mass spectrometric analysis of WR leg muscle was carried out [15]. In order to determine potential protein-protein interactions of the mass spectrometrically identified proteins with an altered concentration in atrophying WR muscle, the publically available STRING (http://string-db.org/; version 9.1) database was used, which contains known and predicted protein interactions including direct physical and indirect functional protein associations [118]. The resulting interaction map revealed how complex the protein interactions patterns are between the affected protein species in WR muscle (not shown). This is especially striking with respect to interaction nodes containing contractile elements, cytoskeletal proteins, metabolic enzymes and molecular chaperones. A significantly increased abundance was shown for myosin light chains, the troponin subunits TnI, TnC and TnT, and the regulatory element tropomyosin, as well as aldehyde dehydrogenase, histone protein H4, the nuclear envelope protein lamin, fatty acid binding protein, fructose-1,6-bisphosphatase, four-and-a-half LIM domains protein 1, carbonic anhydrase CA3 and annexin. Higher levels of heat shock protein beta-1, peptidyl-prolyl cis-trans isomerase A and the small heat shock protein αB-crystallin suggested an elevated cellular stress response in WR muscle [15]. The previous immunoblot analysis of essential Ca^{2+}-binding proteins demonstrated that the luminal Ca^{2+}-shuttling protein sarcalumenin of the longitudinal tubules and the highly abundant Ca^{2+}-buffering protein calsequestrin of the terminal cisternae are increased in WR muscle [15]. In contrast, the cytosolic Ca^{2+}-binding protein parvalbumin is decreased in atrophying fibres [15, 16]. These complex proteome-wide alterations suggest that distinct differences exist between disease-related muscular atrophy and the above outlined changes due to muscular disuse or experimental denervation. Differential changes in proteins associated with glycolytic or oxidative mitochondrial metabolism indicate that WR muscle does not undergo a unidirectional transition towards a particular bioenergetic pheno-

type. In motor neuron disease, the metabolic weighting of bioenergetic pathways and fibre type specification appears to be influenced by at least two main mechanisms. Firstly, a differing degree of a subtype-specific vulnerability of neuromuscular synapses and secondarily, highly complex patterns of compensatory mechanisms of fibre type shifting. Thus, in contrast to an overall slow-to-fast transformation process during muscular disuse or denervation, motor neuron disease seems to be related to changes in both slow and fast isoforms of muscle marker proteins.

5. Concluding Remarks

Pathobiochemical insights from label-free mass spectrometry and fluorescence 2D-DIGE analysis are of considerable interest to the field of muscle pathology, but are also crucial for furthering the discovery of specific protein biomarkers of ALS. Figure 4 summaries the main groups of muscle-associated proteins changed in the WR model of motor neuron disease as recently determined by proteomics. Interesting new findings are the identification of increased levels of certain mitochondrial proteins, suggesting that muscular atrophy in WR muscle is not directly linked to slow-to-fast fibre type shifting, but probably initially involves a preferential loss of neuromuscular synapses that function under normal conditions within a fast type of innervation process. The increased concentration of a variety of heat shock proteins agrees with the idea of extensive cellular stress in ALS and the continuous requirement to remove or re-fold large numbers of affected proteins. A promising new muscle-associated biomarker of motor neuron disease is the fast isoform of MBP-C. A decrease in this major auxiliary protein of the contractile apparatus was demonstrated by the fluorescence 2D-DIGE technique [16], LC-MS/MS analysis [15] and the transcriptomic screening of ALS muscle biopsies [49], making this filament-associated component a suitable biomarker candidate of ALS. The future screening of large numbers of ALS patient samples will be needed to fully establish the newly identified proteomic markers as reliable indicators of disease-related muscular atrophy [119]. Hopefully a few of these proteins will be exploitable to design superior prognostic and therapy monitoring assay systems consisting of a meaningful and diagnostically conclusive biomarker signature. In the future, it can be expected that biomarkers of ALS will play a prominent role for a more accurate diagnosis, the proper monitoring of muscular atrophy and the determination of clinical outcome measures.

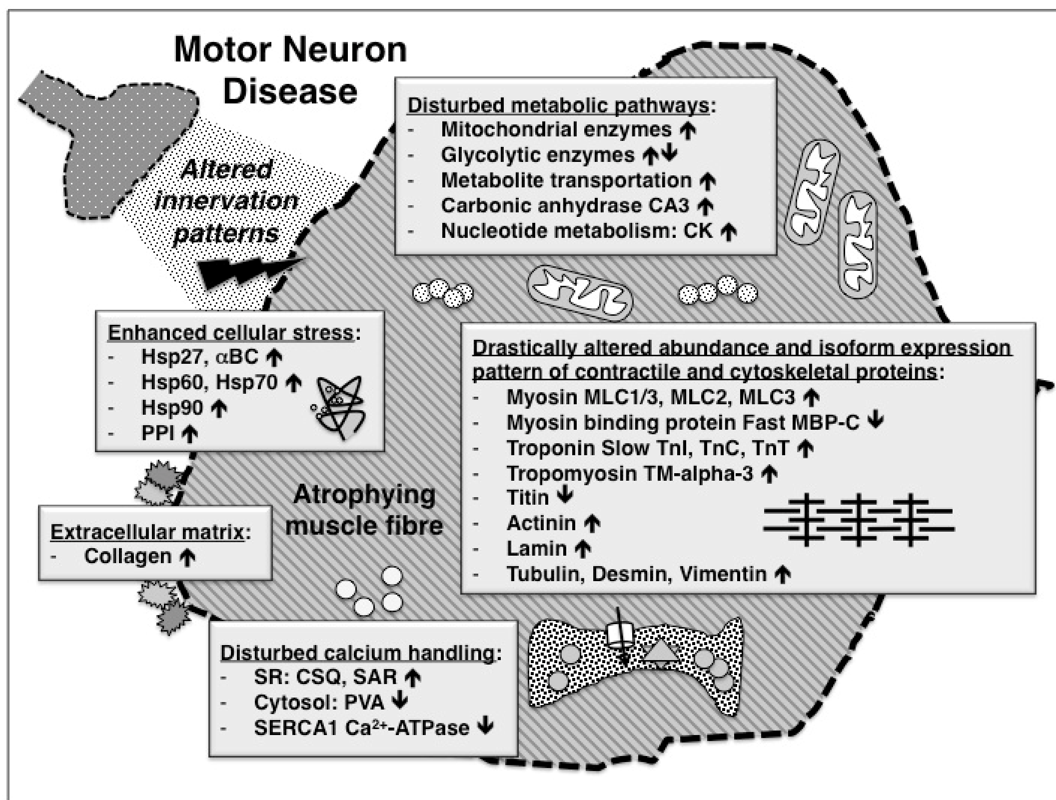

Figure 4. *Overview of major pathobiochemical changes in motor neuron disease as revealed by mass spectrometry-based proteomics.* Listed are subcellular regions, metabolic pathways, cellular processes and protein families that are majorly altered in skeletal muscles due to progressive neurodegeneration. Various newly identified muscle-associated proteins with a changed abundance may be useful as novel biomarker candidates for the design of improved diagnostic, prognostic or therapy monitoring assay systems.

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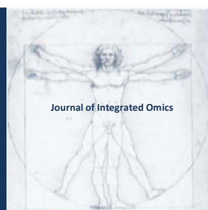

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Quantitative mass spectrometry of urinary biomarkers

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Abstract

The effectiveness of treatment of renal diseases is limited because the lack of diagnostic, prognostic and therapeutic markers. Despite the more than a decade of intensive investigation of urinary biomarkers, no new clinical biomarkers were approved. This is in part because the early expectations toward proteomics in biomarkers discovery were significantly higher than the capability of technology at the time. However, during the last decade, proteomic technology has made dramatic progress in both the hardware and software methods. In this review we are discussing modern quantitative methods of mass-spectrometry and providing several examples of their applications for discovery and validation of renal disease biomarkers. We are optimistic about future prospects for the development of novel of specific clinical urinary biomarkers.

Keywords: quantitative mass-spectrometry; proteomics; urinary biomarkers; renal disease.

Abbreviations

MS: mass spectrometry; **2DE**: two dimension gel electrophoresis; **MALDI-TOF**: matrix-assisted laser desorption ionization-time-of-flight; **2D-DIGE**: two dimension differences gel electrophoresis; **LC-MS/MS**: liquid chromatography tandem mass spectrometry; **SILAC**: stable-isotope labeling by amino acids; **iTRAQ**: isobaric tags for relative and absolute quantification; **SCX**: strong cation exchange chromatography; **ERLIC**: electrostatic repulsion:hydrophilic interaction chromatography; **PAI**: protein abundance index; **SRM**: selected reaction monitoring; **MRM**: multiple-reaction monitoring; **AKI**: acute kidney injury; **Cr**: creatinine; **BUN**: blood urine nitrogen; **CKD**: chronic kidney disease.

1. Introduction

Currently the effectiveness of treatment of renal diseases is limited by the lack of diagnostic, prognostic, and therapeutic markers. A renal biopsy is often necessary to establish a diagnosis, particularly in the case of glomerular diseases. Renal biopsy is a highly invasive method associated with high morbidity and mortality. In contrast, urine is an easily accessible biofluid and its protein content is derived mainly from the kidney and low urinary tract organs. Thus, urinary biomarkers are an attractive tool for development of clinical tests. Recently mass-spectrometry (MS) is playing an increasing role in the identification and quantification of biomarkers [1-6]. Despite its promise, the translation of urinary biomarkers into the clinic has been inefficient [7]. Part of the problem can be attributed to the underestimating of efforts required to discover novel biomarkers and underdevelopment of MS technology. There are several major obstacles for the development of clinically relevant urinary biomarkers [8]. Both the nature of urine and the MS techniques are responsible for generation of non-reproducible results. There is no standard protocol for urine collection and storage, concentration of samples, protein isolation and sample preparation for MS [9-11]. Urine has a high level of variability in volume and protein concentration. Urine composition depends on diet, circadian rhythms, age, gender and exercise [11-15]. Because MS-based methods are very sensitive and capable of detection of femtomoles of peptides, different methods for urine collec-

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tion, concentration and protein isolation can yield distinct proteins discovery [16-18]. This question was intensively studied and discussed during a last decade, and it is not a major subject of this review. Human Proteomics Organisation (HUPO) has developed guidlines for patient data recording, urine collection and sample preparation for several MS based methods (http://www.hupo.org/initiatives/human-kidney-and-urine-proteome-project-hkupp/). A standard method still not commonly used by the proteomics community and most studies use protocols developed to their specific experiments. Urine samples can be diluted or concentrated depending on their water content, thus requiring normalization of biomarker concentrations. The most common normalization factor is urine creatinine (Cr), but its urinary concentration may vary depending on the level of muscle Cr generation (muscle mass) and renal tubular Cr secretion [19, 20]. The muscle mass depends on age, gender, race, fitness and muscle disease, and normalization of urinary samples using Cr can increase protein concentration variability even in the samples collected from healthy individuals. Specific gravity has also been used for normalization of urine samples [21]. Specific gravity is the ratio of the weight of a solution to the weight of an equal volume of distilled water. It is strongly influenced by both the number of particles in the solution and their size. Normalization of urinary proteins using specific gravity is problematic when large molecules are present in urine. Thus the best method for urine normalization is still under investigation. Because debris of spontaneously dying renal cells is released into the urine, uncontrolled amounts of intracellular and membrane proteins can be detected especially in highly concentrated samples collected from the patients with epithelial cell injury. Recently, urinary exosomes were used as a potential source of biomarkers of renal diseases [22-25]. Exosomes are low density inverted apical membrane vesicles normally secreted into the urine from all parts of nephron [22]. They are smaller than apoptotic vesicles, and can be separated from them by gradient centrifugation. They have been found to contain many disease-associated proteins including aquaporin-2, polycystin-1, podocin, non-muscle myosin II, angiotensin-converting enzyme, Na+ K+ 2Cl- cotransporter, thiazide-sensitive Na-Cl cotransporter, and epithelial sodium channel [22]. Exosomes may be usefull for determination of biomarkers for renal dysfunction and structural renal disease [23]. However, the lack of standard efficient methods for vesicle isolation and lysis, and the issue of protein normalization are major limitations for the quantitative proteomics of exosomes [26-28].

Despite all these shortcomings, urine is an attractive source of renal diseases biomarkers because of its noninvasiveness, large volume and because its proteins are originated from the kidney and low urinary tract organs.

In recent years the increased capability of the quantitative proteomics was based on the advances in both hardware and software methods. The increased performance capabilities, easy operation, and robustness of MS over other techniques have made it an ideal platform for quantitative proteomics. Novel MS-based quantitative methods offer the opportunity for faster, higher throughput, and a wider dynamic range protein analysis, and can be used for both stable-isotope labeling or label-free methods of protein quantification. While several quantitative proteomics approaches exist, each of them has its own advantages and limitations. In this review, we discuss modern quantitative proteomics approaches and their applications for the discovery and validation of urinary biomarkers of renal diseases. We do not describe all urinary biomarkers found by particular MS method but rather concentrate on modern quantitative MS methods and their application for urine proteomics. For each MS method we described only few examples that highlight the usefulness of it for urinary proteomics research.

2.1 Two-dimension gel electrophoresis (2DE)

The 2DE method is a primary technique that has been widely used in urinary proteomics [29-31]. In this gel-based method, urinary proteins are resolved in the first-dimension based on their isoelectric point (pI) followed by resolution based on molecular weight in the second-dimension. The gels are then stained by either Coomassie Brilliant Blue, silver stain or Sypro Ruby fluorescent stain to visualize the protein spots. The important step before the gel separation is urine concentration. Multiple protocols have been developed to concentrate and purify urinary proteins including lyophilization, precipitation, ultracentrifugation, and centrifugal filtration [11, 18, 29, 32-35]. Analysis of 2DE images is performed using computer-based platforms. Several commercial programs became recently available including *Melanie* (Geneva Bioinformatics), *ImageMaster2D* (GE Healthcare), *PDQuest* (Bio-Rad Laboratories), *Dymension* (Syngene), *SameSpots* (Totallab), *BioNumerics* (Applied Methods) and *Delta2D* (Decadon). The main steps in differential analysis of 2DE gels involve image noise substraction, protein spot detection, spot quantification, spot matching and statistical analysis. Most programs first detect spots, estimate spot boundaries, and calculate spot volumes for each individual gel, and then match the detected spots across different gels. This procedure may lead to spot mismatching and missing data, which require manual editing of data. Manual editing significantly increases time of analysis, decreases throughput and compromises the objectivity and reproducibility of the analysis [36]. Several novel software such as SameSpot (Totallab) and Pinnacle align the images before processing to reduce spot missmatching [37]. It significantly reduces time of analysis and increase reproducibility. After quantification analysis protein spots are extracted from the gel and identified by mass spectrometry (peptide mass fingerprinting) [38]. Matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry and electrospray ionization (ESI)-MS are most often used for the identification of the extracted proteins. This approach could lead to separation and identification of about 2000 unique spots [34,

39]. This approach was successfully used for identification of potential biomarkers of different renal diseases. High urinary levels of β2-microglobulin, retinol-binding protein, transferrin, hemopexin, haptoglobin, lactoferrin, and neutrophil gelatinase-associated lipocalin (NGAL) were identified as candidate biomarkers for HIV-associated nephropathy [40]. Retinol-binding protein was also identified as a candidate biomarker for acute tubular necrosis [41]. Retinol-binding protein 4, α-1-microglobulin, zinc-α2 glycoprotein, and α-1B glycoprotein were found to increase in the samples from micro-albuminuric patients with type 1 diabetes [42].

However, 2DE method has multiple limitations. Both the separation and the analysis are time consuming reducing number of urine samples. Gel to gel variability reduces reproducibility, and requires complex image analysis and manual correction. Importantly, because quantification of proteins is performed on the basis of in-gel proteins staining, it depends on the sensitivity of particular stain. The sensitivity of Coomassie Brilliant blue is about 50 ng of protein per spot or 20 ng per spot for colloidal Commassie Blue. Additional variability of results arises from destaining procedure and high background. The sensitivity of silver stain is higher than Coummassie Blue (about 1 ng per spot) but both stains demonstrate poor linear response. Sypro Ruby stain demonstrated similar with silver stain sensitivity (about 1 ng per spot) but less background and good linear response for various protein concentrations. But the sensitivity of in-gel methods is thousand times lower than sensitivity of MS-based methods. Thus low reproducibility and low relative quantification accuracy are additional obstacles [43]. Also, 2DE has a small dynamic range compared to MS-based methods being mostly suitable for major proteins. Though 2DE has its limitations, it remains a popular method of urinary protein analysis because of its robustness, simplicity and availability in most facilities [44, 45]. Moreover 2DE allows separating and studying proteins isoforms, modified proteins and degradated peptides specific for urine that is difficult to do by MS-based methods.

2.2 Two-dimensional difference gel electrophoresis (2D-DIGE)

The 2D-DIGE method is an improved version of 2DE. In this method, two different protein samples (control and a disease) and one internal control (pooled mixture of controls and disease samples in equal proportion) are labeled with three different fluorophores: Cy2, Cy3, or Cy5 before in gel separation. These fluorophores have the identical charge and molecular mass but unique emission wavelengths that allows identification of those fluorophores using appropriate optical filters [46-48]. The labeled samples are then mixed together and separated on a 2DE. The same internal control is used for all samples for normalization. The gel is scanned at three different wavelengths: 488 nm (Cy2), 532 nm (Cy3), and 633 nm (Cy5) and relative abundance of proteins are quantified using computer software such as *DeCyder* (GE Healthcare Life Science), *Melanie* (Geneva Bioinformatics) and *PDQuest* (Bio-Rad). The sensitivity for each fluorescent dye is similar to Sypro Ruby fluorescent dye (about 1 ng per spot). Addition of internal standards to each gel allows protein normalization and quantification of protein amounts as ratios and not as volumes. This method reduces gel-to-gel variation and separates experimental variability form biological one. The quantification accuracy of 2D-DIGE is higher than 2DE method. This technique has been routinely used for the discovery of candidate urinary biomarkers of renal disease in patient and animal models [49-52]. 2D-DIGE-SELDI-TOF (surface-enhanced laser desorption ionization – time of flight) was used for the detection of early stage tubular injury in canine model of progressive glomerular disease [50]. Alpha 1 antitrypsin was discovered as a diagnostic biomarker for diabetic nephropathy [52]. A number of highly abundant proteins in urine such as albumin fragments have also been identified by gel-based proteomics approaches, and these abundant proteins were considered disease-biomarker candidates [53-55]. Major limitations of this method are time-consuming separation and analysis steps that restricts its use used for high throughput screening. When the number of urine samples is large, cost of fluorescent dyes is also an additional limitation. Both 2DE and 2D-DIGE methods have less sensitivity and small dynamic range compared to MS-based methods and are mostly suitable for major proteins.

While 2DE and 2D-DIGE methods employ in-gel quantification based on the protein staining techniques, all other methods described below are MS-based quantification techniques (see Table 1).

3.1 Stable-isotope labeling by amino acids (SILAC)

This method is based on metabolic labeling of proteins with heavy isotopes (H^2, C^{13}, and N^{15}) incorporated into amino acids [56]. A number of amino acids such as arginine, leucine, and lysine with stable isotope are suitable for use in SILAC, but lysine and arginine are the most often used amino acids, because trypsin-digested peptides contain at least one arginine or lysine making all peptides eligible for quantification [57, 58]. Originally this method was developed for *in vitro* cell culture [56]. In this method either two different lines of cells (experimental and control) are cultured under similar conditions with addition of labeled amino acid to experimental cell line, or cells are cultured under different conditions with addition of labeled amino acids to experimental group. Cells are collected after five to seven passages to ensure >95% labeling, lysates are prepared, and then experimental and control samples are combined in a 1 : 1 stoichiometric ratio [56]. Combined samples are separated either on 1DE or 2DE following by in-gel digestion, peptides extraction and LC-MS/MS analysis. Alternatively, the samples are digested in-solution and analyzed by LC-MS/MS. Labeled amino acid induces a shift in the mass/charge (*m/z*) ratio comparing to the unlabelled amino acid. This shift al-

Table 1. Quantitative methods to analyze urinary biomarkers

Method	Quantification	Advantages	Limitation
		Biomarkers Identification	
2DE Two-dimension electrophoresis	In-gel Coomassie Brilliant Blue, silver staining or Sypro Ruby	Robust, simple, cheap. Suitable for protein isoforms, modifications and degradation analysis.	Low reproducibility and relative quantification accuracy, small dynamic range
2D-DIGE Two Dimension Differences Gel electrophoresis	In gel fluorescence intensity of Cy2, Cy3 and Cy5 fluorophores	Reduces gel-to-gel variation and enhances sensitivity Suitable for protein isoforms, modifications and degradation analysis	Variability in labeling efficiencies, small dynamic range comparing to MS based methods, expensive
SILAC Stable-isotope labeling by amino acids	MS based on metabolic labeling of proteins with heavy isotopes *in vivo*	Independent of the degree of resolution and instrument sensitivity, accurate for low abundant protein	Difficult and time-consuming to establish, expensive, complicated data analysis, not suitable for human samples
iTRAQ Isobaric Tags for Relative and Absolute Quantitation	MS based on *in vitro* peptides labeling with eight isobaric tags	Eight samples can be pooled and relative abundance can be quantified in one MS/MS run	Variability in labeling efficiencies, loss of peptides during chromatography, expensive
Label-free method	MS based on peptide peak areas and the spectral counting	High throughput, cheap, simple in sample preparation, less complicated MS analysis.	Less accuracy than tag methods, semi quantitative in nature, not suitable for low abundant and short proteins.
		Biomarkers Validation	
SRM and **MRM** selected reaction monitoring and multiple-reaction monitoring	MS-based on counting the ions for transition pairs	Good linearity and excellent precision, wide range	Targeted approach focused on a limited set of pre-detected proteins

lows to discriminate peptides between experimental and control samples, and to quantify relative changes in protein concentration (Fig. 1 A). Combining the differentially labeled samples before any purification and fractionation steps minimizes the possible quantitative error caused by handling different samples in parallel [56].

Recently, this method has been extended to the animal models [59-61]. Feeding mice with diet containing a heavy isotope C^{13}-lysine for one generation leads to a complete exchange of the natural (light) isotope (12)C6-lysine. Blood, tissue, and organs are labeled, and can be used for global proteomics [62-64].

Additionally SILAC can be used for an indirect 'spike-in' approach where cell line is used to produce a heavy-labelled reference sample, which is added as an internal standard to the tissue or organ samples [65].

SILAC's advantage is that this method does not require a targeted analysis of specific proteins or peptides because every peptide is labeled and can be quantified independently of the degree of resolution and instrument sensitivity. It is also more robust and accurate than other quantitative techniques such as iTRAQ and label-free method [66]. However, SILAC also has several disadvantages. It is difficult and time-consuming to establish this method in new model organisms. The medium composition has to be controlled and the reagents are expensive. The data analysis is also challenging due to incomplete incorporation of labeled amino acids and arginine-to-proline conversion by arginase [67]. Because arginase II is highly expressed in renal cells, labeled proline incorporation into the proteins increases complexisity of data analysis. Moreover, SILAC cannot be used directly in human samples and has not been used for discovery of human urinary biomarkers.

Investigation of renal cell secretome is a potential step in the urinary biomarker discovery. Treatment of HEK-293 renal cells with cyclosporine demonstrated up-regulation of secreted cyclophilins A and B, macrophage inhibition factor and phosphatidylethanolamine-binding protein 1 [68]. Recently, the SILAC-labeled mouse serum was used for 'spike-in' quantification of human serum and urine [69]. SILAC mouse serum was mixed with human serum and urine, and multidimensional separation and LC-MS/MS analysis was performed. The shared peptides between two species were quantified by SILAC pairs. Analysis of urine from immunoglobulin A nephropathy patients identified novel biomarker candidates, such as Complement C3, Albumin, VDBP, ApoA1, and IGFBP7 [69]. Thus, despite the fact that SILAC cannot be used directly in human samples, its application in

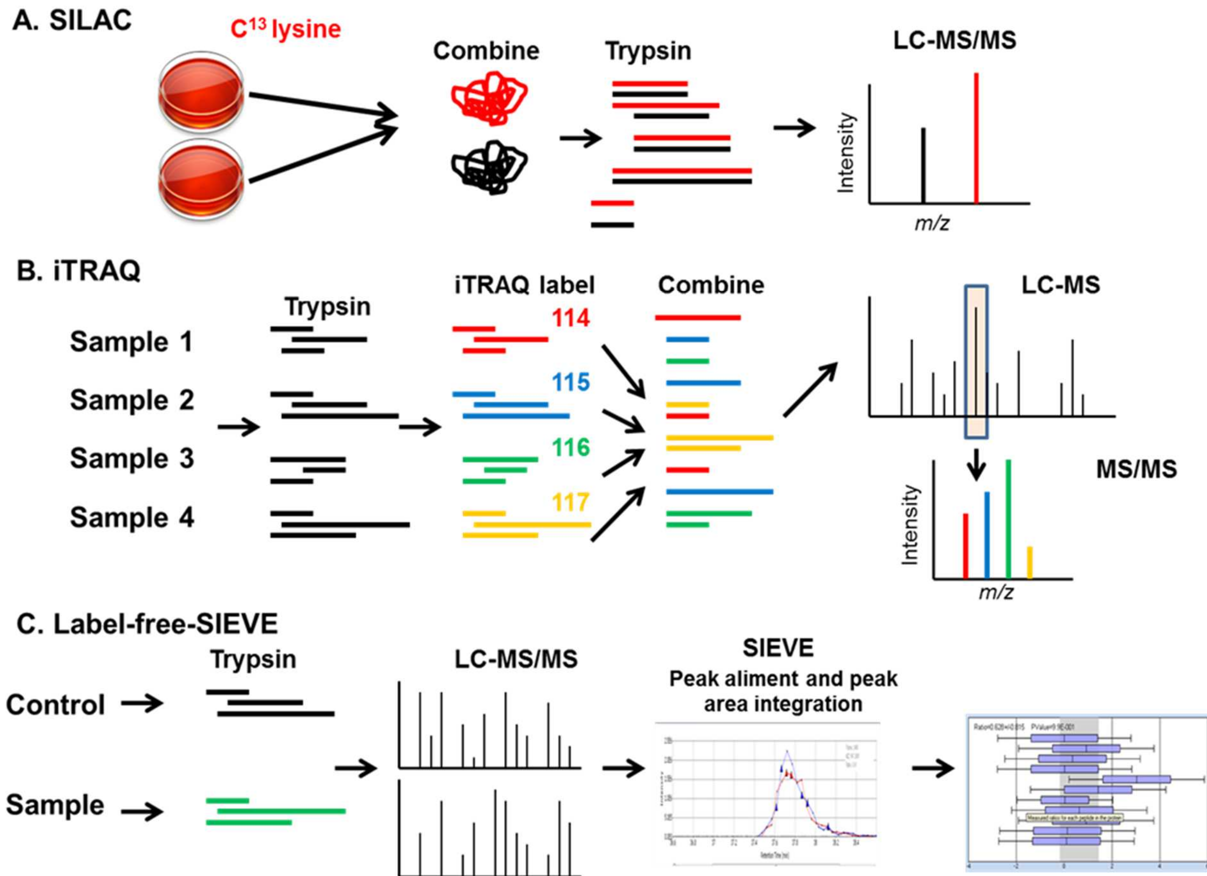

Figure 1. *MS- based quantification methods*. (**A**). SILAC- Stable-isotope labeling by amino acids. Cells are differentially labeled by growing them in medium with normal lysine (black color) or with heavy lysine (C^{13}-lysine, red color). Both samples are combined, trypsinized and LC -MS/MS is performed. Metabolic incorporation of the amino acids into the proteins results in a mass shift of the corresponding peptides. (**B**) iTRAQ- Isobaric Tags for Relative and Absolute Quantitation. Samples are trypsinized, and peptide are labeled *in vitro* with iTRAQ tags with different mass (114-red, 115-blue, 116, green and 117-yellow color). Samples are combined together and LC-MS/MS is performed. Identical peptides labeled with the different iTRAQ tags produce the same peak in MS spectra (shown in rectangle). MS/MS fragmentation of ion produces unique peak for each tag that allowed comparison of relative intensity. (**C**) Label-free quantification using SIEVE program. Sample (red color) and control (blue color) are processed separately and LC-MS/MS is performed. SIEVE program from Thermo Electron perform aliment of peaks, peak area integration and spectral counting, that quantify relative amount of protein in sample.

renal cell secretome and animal models can potentially lead to the discovery candidates biomarkers of renal disease.

3.2 Isobaric Tags for Relative and Absolute Quantitation (iTRAQ)

iTRAQ is a method of *in vitro* peptides labeling after trypsin digestion of proteins that allowed to compare multiple samples in one MS/MS run [70-72]. iTRAQ label consists of a reporter group with a defined molecular weight, a balance group, and an amine-reactive group that reacts at lysine side chains and NH_2-terminal amino acid. Recently, eight iTRAQ reagents became available, with the following reporter/ balance group masses: 113 / 192, 114 / 191, 115 / 190, 116 / 189, 117 / 188, 118 / 187, 119 / 186, and 121 / 184 Da. The combined mass remains constant (305 Da) for each of the eight reagents. The iTRAQ labels are generated using heavy weight isotops of ^{13}C, ^{15}N, and ^{18}O atoms in such way that all peptides with different iTRAQ labels attached are isobaric (same mass) and indistinguishable in chromatographic separation and MS. The function of balance groups is to make all iTRAQ tags isobaric so the combined mass of reporter group and balance group remains constant. Following fragmentation in MS/MS the iTRQ label looses the balance group, while the charge is retained by the reporter group. The eight reporter group ions appear as distinct masses in MS/MS that can be used to identify and quantify individual members of the multiplex set [70]. In iTRAQ, up to eight (8-plex) samples are labeled after trypsin digestion with iTRAQ reagents. The samples then are pooled together, the labeled peptides are separated by strong cation exchange chromatography, and the isolated labeled peptides are separated by LC-MS/ MS [73]. Different samples can be run together in the single MS/MS run. The isobaric nature of the tags allows the pro-

tein samples to be pooled together after labeling without increasing the complexity of the MS analysis. Identical peptides labeled with the different iTRAQ reagents produce the same peak (ion) in MS spectra. Upon MS/MS fragmentation of the parent ion, unique signature ions are generated which distinguish the individual samples and allow to compare the relative amount of each sample (Fig. 1B). iTRAQ method can also be used for absolute quantification of peptides by adding an internal standard peptide. The advantage of iTRAQ labeling is that the signal obtained from combined peptides enhances the sensitivity of detection in MS/MS. However, the variability in labeling efficiencies and the costly reagents are major limitations of this method [74]. Labeling also increases complexity of the samples and can reduce number of the identified peptides during MS/MS run. Some peptides are lost during the separation on SCX chromatography. Recently, electrostatic repulsion-hydrophilic interaction chromatography (ERLIC) have been developed as an alternative to the SCX chromatography [75]. ERLIC method separates peptides on the basis of electrostatic repulsion and hydrophilic interaction and is found to increase the proteome coverage.

The use of this powerful technique is gradually becoming the method of choice in the field of biomarker discovery [3, 76-78]. This method allowed discovering P- and E-cadherins as urinary biomarkers of idiopathic nephrotic syndrome [76]. Alpha-1-antitrypsin, alpha-1-acid glycoprotein 1, and prostate stem cell antigen has been discovered as candidate biomarkers for diabetic nephropathy [77]. Uromodulin, SERPINF1, and CD44 were identified and verified in an independent cohort as urinary biomarkers to differentiate patients with early acute kidney transplant rejection from other groups [78].

3.3 Label-free quantitative methods

To overcome the problems in the labeling techniques such as high cost of the reagents, higher concentration of sample requirement, and incomplete labeling, label-free shotgun proteomic technologies have been developed. These methods are based on the assumption that the peak area of a peptide in the chromatogram is directly proportional to its concentration [79-81]. Label-free protein quantification approach is based on two types of measurements; the measurement of ion intensity by quantification of peptide peak areas or peak heights in chromatogram, and the spectral counting in the MS/MS analysis. For spectral counting, peptides from the same protein are identified, chromatographic peaks aligned and normalized (Fig.1C). There are several commercially available software packages for label-free analysis (*Decyder MS* from GE Healthcare, *Protein Lynx* from Waters, and *SIEVE* from Thermo Electron). This approach is primarily used for the analysis of human samples and has been applied to the analysis of urinary proteome [1, 82, 83]. It is a very high throughput technique that increases opportunities in the discovery of candidate biomarkers. There are several advantages in label-free quantification approach. It is a cheap method comparing to the labeling techniques. It is simpler in terms of sample preparation, and less complicated in terms of MS/MS analysis [81]. The limitation of this method is redundancy in peak detection which arises from the peptides which are similar for several proteins [84]. Other limitations of label-free quantification methods are less accuracy, semi-quantitative nature, and unsuitability for low abundance and small proteins [85]. Small proteins or proteins of low abundance could still be present in the sample in spite of the spectral count being zero, larger proteins generate more tryptic digest products, and more spectral counts. Another limitation of the method is a spectra normalization. In contrast to SILAC and iTRAQ methods, in label-free method the spectra are generated in separate MS/MS runs that are different in many factors like efficiency of fragmentation and ionization [85]. Label-free quantification methods overcome those limitations by additional computational calculations. There are several algorithms available that take into account the sequence and length of the peptides and compute the predicted abundance of proteins in the sample [86-88]. Protein abundance index (PAI) is defined as the number of identified peptides divided by the number of theoretically observable tryptic peptides for each protein. Absolute quantification of proteins is based on exponentially modified PAI values with or without added standards [79, 85].

Label-free quantitative analysis of urinary exosomes in diabetic nephropathy resulted in the discovery of three proteins AMBP, MLL3 and VDAC1 as candidate biomarkers [24]. Another group of proteins (Tamm-Horsfall glycoprotein, progranulin, clusterin and α-1 acid glycoprotein) were determined as candidate biomarkers for microalbuminuria progression in diabetic nephropathy [89].

4. MS-based absolute quantification methods for biomarkers validation

The methods described above have been used mostly for urinary biomarkers discovery. Traditional methods such as Western blot and Elisa are the first choice for validation of biomarkers, but novel stable isotope dilution MS (SID-MS) quantification methods suitable for validation have been developed. Two methods (selected reaction monitoring (SRM), and multiple-reaction monitoring (MRM)) have been used for absolute quantification of proteins in combination with stable isotope dilution. These methods are based on the addition of known quantities of isotope-labeled standards, which have similar chromatographic properties to the target compounds but can be distinguished from them by their difference in m/z [90, 91]. The isotope dilution method is a targeted approach focused on a limited set of proteins. The identification of candidate proteins requires the prior generation of isotope-labeled standards [92, 93]. Quantification is performed by comparing the peak height or peak area of the isotope-labeled and the native forms of a

peptide of interest. SRM is a non-scanning mass spectrometry technique, performed on triple quadrupole instruments. In SRM experiments, two mass analyzers are used as static mass filters, to monitor a particular fragment ion of a selected precursor ion. The specific pair of *m/z* values associated with the precursor and fragment ions selected are referred to as a "transition" [94]. Unlike common MS based proteomics, no mass spectra are recorded in a SRM analysis. Instead, the detector acts as a counting device for the ions matching the selected transition thereby returning an intensity value over time. In MRM experiment, multiple transitions can be measured within the same experiment on the chromatographic time scale by rapidly shifting between the different precursor/fragment pairs. Typically, a triple quadrupole instrument cycles through a series of transitions and records the signal of each transition as a function of the elution time.

The major advantage of these methods is good linearity and excellent precision, but the accuracy and ability to determine the true abundance of target protein strongly depends on the choice of selected peptides and the purity of internal standards [95, 96]. This method covers a complete dynamic range of cellular proteome, with a low limit of detection below. 50 copies of protein per cell [97]. The disadvantage of these methods is that they are limited to a small number of proteins because suitable internal standards have to be purchased or synthesized. SID-MS based quantification is filling the gap between the discovery and validation of biomarkers that may promote candidate biomarkers towards clinical trials and established them as diagnostic tools. However, developing and validating SID-MS-based assays is an expensive and time consuming process, requiring a coordinated and collaborative effort by the scientific community through the sharing of publicly accessible data and datasets, bioinformatic tools, standard operating procedures, and well characterized reagents [98].

There are several examples of recent coordinated efforts for development of urinary biomarkers for renal diseases. The Nephrotoxicity Working Group of the Predictive Safety Testing Consortium have selected 23 previously discovered urinary biomarkers and evaluated them in rat models of acute kidney injury (AKI) [99-103]. Seven markers were selected for further preclinical studies, including: kidney injury molecule-1 (kim-1), albumin, total protein, β_2-microglobulin, cystatin C, clusterin, and trefoil factor-3. Chronic kidney disease (CKD) consortium (www.ckdbiomarkersconsortium.org) have identified fourteen candidate biomarkers for CDK progression and twelve biomarkers for early stage CKD in diabetes and lupus nephrology [104, 105]. Future coordinated efforts from scientific community will validate recently discovered biomarkers of renal diseases.

5. Concluding Remarks

Nephrology is in a dire need for improved diagnostic and therapeutic markers. Despite the more than a decade of intensive investigation of urinary biomarkers no new clinical biomarkers were approved [106]. Similar to the early genomic studies, expectations toward proteomics in biomarkers discovery were significantly higher than the ability of the technology a decade ago. The technology was underdeveloped with limited analytical and quantification capability. Thus early investigations in this area were largely confined to measurement of major urinary proteins without association with disease mechanisms. Now it is clear that the most promising biomarkers have been found in well-designed studies guided by specific research questions. Moreover, during the last decade, proteomic technology has made dramatic progress in both the hardware and software methods [107]. Advances in quantitative proteomics and development of SRM and MRM methods let the protein-quantification data stand by their own without validation from other protein quantification methods as Western blot and Elisa [108]. This progress opens a new era in the discovery and validation of urinary biomarkers of renal disease. Collaborative efforts by the scientific community are needed for the development of standardized protocols for sample preparation methods suitable for examination of low-abundance urinary proteins. Addition of other indirect approaches, such as cell cultures and animals models, may be useful for the discovery of potential biomarker candidates that could be subsequently found in urines. Uncovering of disease molecular mechanisms may predict new candidate urinary biomarkers.

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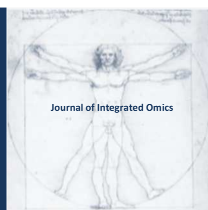

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Characterization of temperature-sensing and PIP_2-regulation of TRPV1 ion channel at the C-terminal domain using NMR spectroscopy and Molecular Dynamics Simulations

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ABSTRACT

Transient receptor potential (TRP) channels are receptors of stimulating signals, such as temperature, taste, odor, and chemo- and mechano-stimuli. Temperature sensing TRP channels coincidently function as pain receptors, and are potential targets for substances of abuse, including alcohol and illicit drugs. TRP vanilloid type 1 (TRPV1) channel is activated by heat (>43 °C) and capsaicin under the tight regulation of membrane-associated second messenger, PIP_2 (phosphatidylinositol-4,5-bisphosphate), responds to noxious stimuli and inflammatory substances, and could potentially modulate effects of alcohol and drugs of abuse. Despite the crucial roles in mediating signal transductions at both peripheral and central nervous systems, TRP channels are poorly understood in the context of structures and mechanisms. In this study, we describe our initial structural characterization of the TRPV1 C-terminal domain, the putative temperature sensing and PIP_2-regulatory domain, using NMR spectroscopy and molecular dynamics simulations. Both experimental and computational models suggest that the C-terminal domain is intrinsically unstructured at room temperature with and without lipid bicelles. Elevated temperature and PIP_2-binding can induce substantial conformational changes and formation of considerable secondary structural components in the C-terminal domain, which could be transduced to the transmembrane domain to potentially sensitize the channel.

Keywords: membrane protein; molecular dynamics; NMR; phospholipids; signal transduction; transient receptor potential channel.

Abbreviations

MD: molecular dynamics; **NMR**: nuclear magnetic resonance; **NOE**: nuclear Overhauser effect; **PIP_2**: phosphatidylinositol-4,5-bisphosphate; **TRP**: transient receptor potential.

1. Introduction

TRP channels are generally described as the vanguard of our sensory systems that respond to a variety of intra- and intercellular stimuli [1, 2]. The thermo TRP channels are activated by distinct physiological temperatures, and are involved in converting thermal information into chemical and electrical signals within the sensory nervous system. The homologously related TRPV1, TRPV2, TRPV3, and TRPV4 are activated by increased temperature, while TRPM8 and TRPA1, the more distinctly related TRP channels, are activated upon cooling [3, 4]. In addition to temperature, these channels can be activated by a number of agonists. For example, capsaicin, the pungent extract of hot peppers, can activate TRPV1; menthol, the cooling compound extracted from the mint plant, directly activates TRPM8; and mustard oil and cinnamaldehyde from the cinnamon oil specifically activate TRPA1 [2, 4].

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Coincidently, thermo TRP channels are receptors of noxious stimuli, leading to acute nociceptive pain, a protective warning of damage [5, 6]. However, many pathological conditions lead to changes in the expression level and/or sensitivity of nociceptive TRP channels, characterized as hyperesthesia. These pathological conditions include inflammations (inflammatory pain) and damage or lesions to the nervous system (neuropathic pain) [7]. Recent research also suggests that alcohol can modulate thermo TRP channel activities. TRPM8 activity is inhibited by high concentration of ethanol [8], while TRPV1 and TRPA1 are activated and potentiated by ethanol [9-11]. Using TRPV1 knockout mice, the roles of TRPV1 in the avoidance of the adverse alcohol taste and alcohol-induced intoxication were established [12, 13].

Most recently, high-resolution structures of TRPV1 ion channel were determined using state-of-the-art single particle cryo-electron microscopy (cryo-EM) technique [14], and distinct conformations were revealed upon activation of the channel [15]. These structures contains amazingly detailed information on the arrangement of the transmembrane segments, including the ion passage pore, and the cytosolic N-terminal domain, including the ankyrin repeats. However, the TRPV1 structure (PDB ID: 3J5P) was obtained using a minimal functional construct composed of residues 110-603 and 627-764 [14], which lacks about half of the cytosolic C-terminal domain (residues 684-839). In addition, this structure lacked electron density in the C-terminal region with the exceptions of the TRP domain α-helix and a β-strand close to the end of the minimal construct. This observation strongly suggested a largely unstructured C-terminal domain that could undergo significant conformational change upon activation or ligand binding.

The intrinsically disordered C-terminal domain of TRPV1 is functionally critical. It has been suggested that the cytosolic C-terminal domains of thermo TRPs are responsible for mediating TRP channel activities. Swapping mutageneses experiments indicated that the C-terminal domains of TRPV1 and TRPM8 determine the activation phenotype by temperature of these channels [16]. It is also determined that a region located outside the TRP domain comprising the TRPV1 C-terminal amino acids Q727 to W752 (corresponding TRPM8 C-terminal amino acids K1030 to W1055) is the minimal portion to show temperature sensitivity (heat or cold), and deletion of 11 residues, comprising of TRPV1 C741 to W752, results in losing channel thermal sensitivity while retaining voltage sensitivity [17]. The minimal construct used to obtain the TRPV1 structure contained this temperature-sensing segment (residues 727 – 752), and the minimal construct was reported to respond to heat [14].

PIP_2 (phosphatidylinositol-4, 5-bisphosphate) is an essential modulator for TRP channels, as well as a wide range of other ion channels [18]. PIP_2 activates TRPM8, and activation of phospholipase C (PLC) and subsequent depletion of PIP_2 desensitize the channel [19, 20]. On the other hand, TRPV1 is desensitized by PIP_2, and depletion of cellular PIP_2 upon activation of PLC activates TRPV1 [21]. However, the role of PIP_2 on TRPV1 modulation is still controversial, as experiments also show that PIP_2 sensitizes TRPV1 and that depletion leads to desensitization [22, 23]. A dual regulatory role is also suggested [24]. It has been suggested that the conserved positively charged clusters in the C-terminal domain of TRP channels are responsible for PIP_2 binding. The putative PIP_2 binding site for TRPM8 is on the very proximal C-terminal TRP domain (K995, R998, K1008) [20], whereas in TRPV1, it is located on the more distal C-terminal region after the TRP domain (R786, K789, R798) [21]. A homology model of TRPV1, built upon the crystal structures of Kv1.2 [25] and HCN2 [26] as templates for transmembrane and C-terminal regions, respectively, suggests that PIP_2 aliphatic chains are located near the voltage-sensor modules, while the PIP_2 polar head group is interacting with a cluster of positive charges located in the proximal C-terminal region, including residues K694, K698, K701, and K710, as well as with amino acids R575 and R579 located in the S4-S5 linker helix [27]. The C-terminal domain in this homology model, however, did not seem to agree with the recent cryo-EM structure, which indicated a mostly unstructured C-terminal domain with an α-helical TRP domain and a β-strand in the temperature sensor [14], while the homology model showed a mostly well-folded C-terminal domain with all helical components within the temperature sensor [27].

Thermo and nociceptive TRP channels have the ability to integrate multiple stimuli, and temperature activation thresholds can be shifted in response to algogenic substances, phospholipid signaling molecules, phosphorylation states, mild acidic conditions, and membrane voltage. A modular model with allosteric gating mechanism of thermo TRP channels was proposed to explain the TRP mechanisms [28]. Current *in vivo* studies on the functions of TRP channels involves mostly the use of transgenic mouse models, providing a productive source of validated targets for future drug discovery [29]. Nevertheless, mouse gene knockouts can be problematic pertaining to pain phenotyping. For example, thermo and pain reception can be compensated by related channels and receptors, and differences between mouse strains can be significant [30]. Moreover, the lack of knowledge on the detailed mechanisms and structures of TRP channels, particularly the C-terminal domain, severely hampers the ability to understand the mechanistic role of TRP channels in nociception and the development of drugs to target TRP channels [31]. TRPV1 is the only member of the TRP superfamily that has been targeted in the treatment of pain, bladder, and gastrointestinal diseases [32]. The minimal construct of the TRPV1 possessed the temperature-sensing segment (residues 727-752), but not the regulatory PIP_2/calmodulin binding segment (residues 778-819), suggesting that the temperature sensing and regulatory segments are functionally independent and structurally unrelated. Considering the intrinsically disordered nature of the C-terminal domain, we have studied these two segments as individual peptides in an initial attempt, and combined with molecular dynamics (MD) simulation of the full-length C-

terminal domain to integrate the findings of the individual peptides into a more complete model. Here we report our initial NMR spectroscopic characterization of the putative temperature-sensing and PIP_2-interacting segments of the C-terminal domain of TRPV1 channel at various conditions, complemented by an MD simulation of the complete C-terminal domain at two different temperatures.

2. Material and Methods

Peptide synthesis

The C-terminal segment that shows temperature sensitivity of human TRPV1 residues 727-752 with sequence QVGYTPDGKDDYRWCFRVDEVNWTTW and the putative PIP_2-interacting segment of human TRPV1 protein residues 778-819 with sequence of LRSGRVSGRNWKN FALVPLLRDASTRDRHSTQPEEVQLKHYT were synthesized and purified at the Proteomics Resource Center of the Rockefeller University, and their identities were confirmed by mass spectrometry.

NMR sample preparation

All NMR samples were prepared in 20 mM sodium phosphate buffer, pH 6.6, 150 mM NaCl, in 90%/10% H_2O/D_2O solvent. The phospholipids, DHPC, DMPC, DMPG, and brain PIP_2 were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). A 15% stock bicelle solution was formed at a molar ratio of 0.53 DHPC:0.27 DMPC:0.20 DMPG in the above buffer [33]. The hTRPV1 727-752 peptide (temperature sensor) was dissolved in the above buffer to a final concentration of 4.0 mg/ml. The mTRPV1 778-819 peptide (PIP_2 interacting segment) was prepared in the buffer and in the bicelle solution in the absence and presence of 4 mol% brain PIP_2. The final peptide concentration in all samples was 3.3 mg/ml and final lipid concentration was 5%.

NMR spectroscopy

All NMR spectra were acquired on a Bruker Avance 900 MHz spectrometer, equipped with a cryogenically cooled TCI-probe, operating at a ^1H frequency of 900.154 MHz. Standard two-dimensional NOESY pulse sequence was used for samples without lipid bicelles, and a ω_2-selective NOESY pulse sequence, in which the final excitation pulse was replaced by an E-BURP2 pulse selective for the frequency region of 6.5 – 10.5 ppm, was used for samples containing lipid bicelles [34]. The NOESY mixing times were set to 100, 200, and 300 ms. 4096×1024 complex data points were collected in each experiment with spectral widths of 11682.243 Hz (or 12.978 ppm) in both dimensions. 16 transients were collected for each 2D increment. The experiments were performed at 25, 35, and 45 °C (298, 308, and 318 K). All spectra were processed using NMRPipe [35], and analyzed and displayed using NMRViewJ [36].

Molecular dynamics simulations

A BLAST search indicated that two proteins are structurally homologous to human TRPV1 C-terminal domain, G684-K839, with PDB ID 2HE7 [37] homologous to the proximal end with sequence identity of 27/109 (25%), similarity of 41/109 (37%), and E-value of 1.3, and 2R5K [38] homologous to the distal end with sequence identity of 19/65 (29%), similarity of 30/65 (46%), and E-value of 4.3. A homology model was built upon these two proteins using program Modeller version 9.11 [39]. The homology model was then minimized and equilibrated using molecular dynamics simulation package NAMD version 2.9 [6] at 298 K and 318 K, respectively. The trajectories were simulated for 10 ns at 2 fs time step using periodic boundary conditions for water solvent under constant temperature/pressure and variable volume conditions. The restart frequency was set to 1 ps (every 500 steps) in the simulations. The structural equilibrations were assessed using graphical presentation tool VMD version 1.91 [40] and the structures were visualized using PyMOL version 1.5 (http://www.pymol.org/).

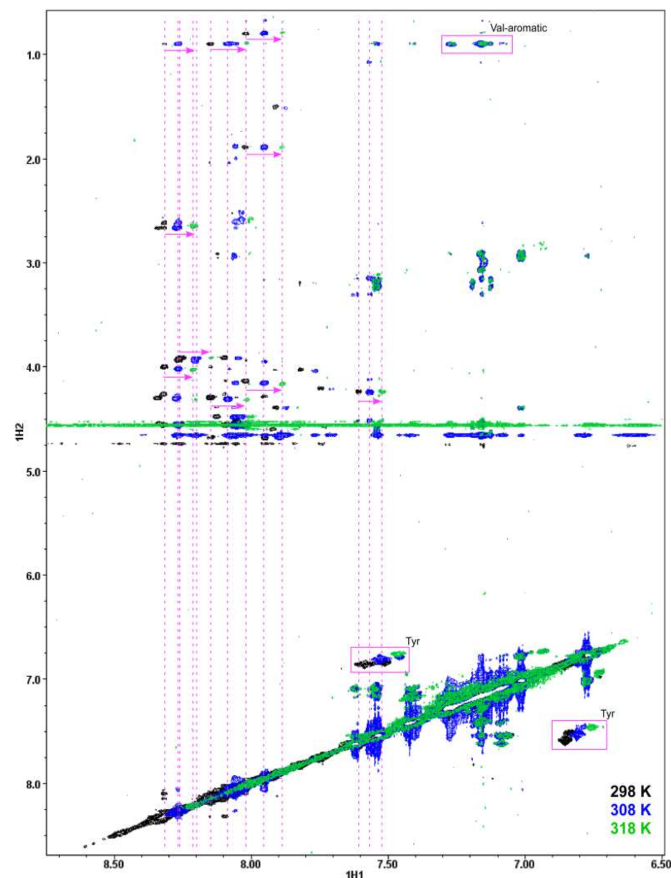

Figure 1. Overlaid NOESY spectra of human TRPV1 residues Q727-W752, the putative temperature sensing segment, at temperatures of 25, 35, and 45 ˚C. The arrows indicate resonance shift of all three valine residues upon increase in temperature, and boxes highlight aromatic-valine interactions and shifts in Tyr resonances in response to temperature change.

3. Results

3.1 *NMR spectroscopy of TRPV1 temperature-sensing segment (Q727-W752)*

The 300-ms mixing time NOESY spectra (amide-aromatic region) of the hTRPV1 temperature-sensing segment, residues Q727-W752, at different temperatures (298, 308, and 318 K) are shown in Figure 1. This segment displays extended, strand-like, secondary structural feature, with inter-chain N-H/N-H (d_{NN}) NOE cross peaks observed at room temperature. Upon increase in temperature, the three valine residues exhibit the most significant shifts, while the single tyrosine residue also shifts continuously. New interactions between valine methyl groups and aromatic residues are appearing only at elevated temperatures (308 and 318 K), whereas the inter-chain d_{NN} NOE cross peaks are disappearing at high temperatures. These observations indicate that upon increase in temperature, the temperature sensing segment experiences significant conformational change, and the hydrophobic core interactions are reorganizing in response to temperature change. The conformational changes at the temperature sensing segment are likely transferred to the transmembrane domain under the regulation at the PIP_2 binding segment.

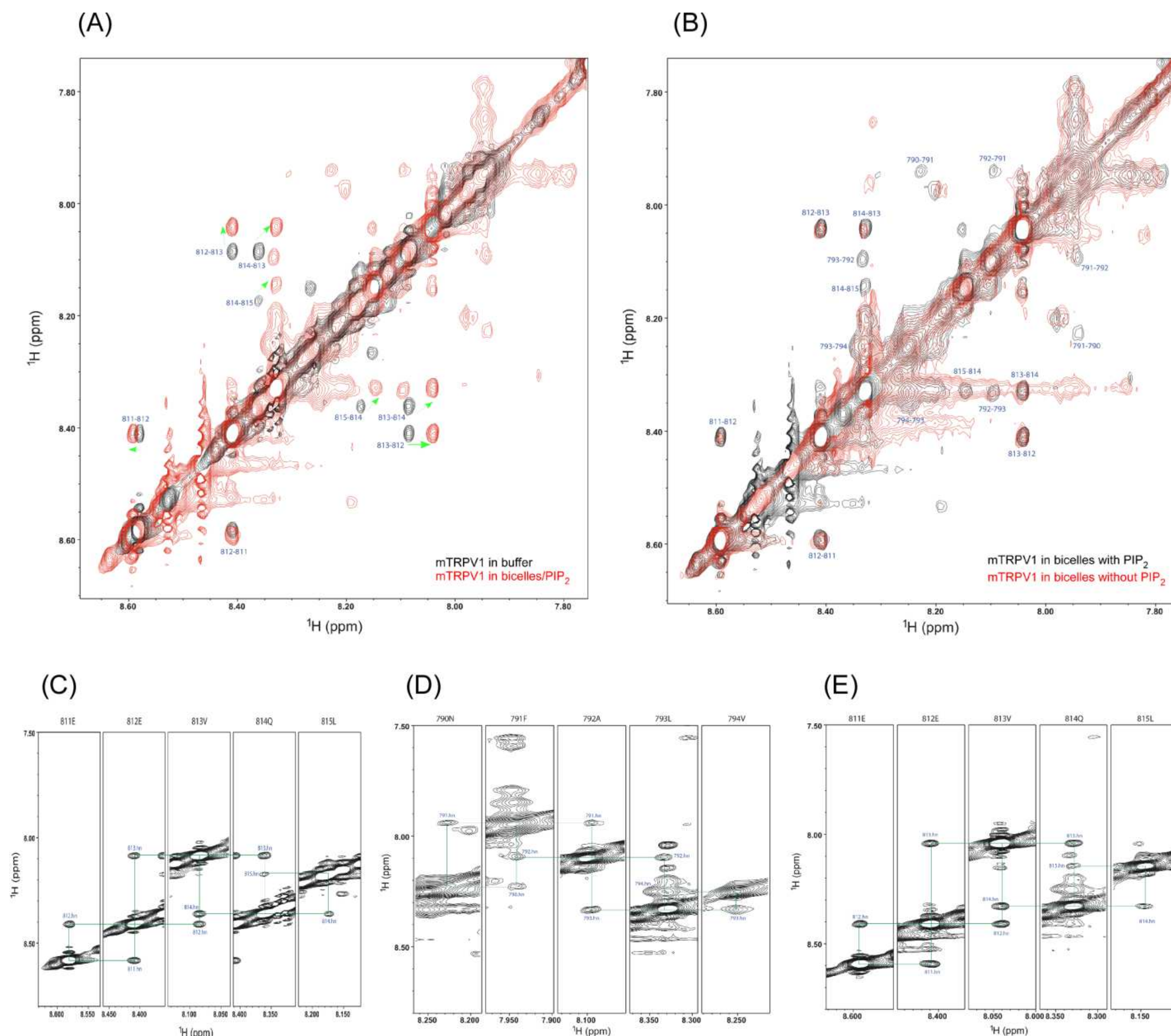

Figure 2. NOESY spectra of mTRPV1 778-819 peptide. (A) Overlay of the d_{NN} region of the peptide in buffer and in lipid bicelles with PIP_2; (B) Overlay of the d_{NN} region of the peptide in lipid bicelles with and without PIP_2; (C) d_{NN} connectivity of the helical stretch 811-815 without bicelles/PIP_2; (D) d_{NN} connectivity of the helical stretch 790-794 in lipid bicelles with PIP_2; (E) d_{NN} connectivity of the helical stretch 811-815 in lipid bicelles with PIP_2.

3.2 *NMR spectroscopy of TRPV1 PIP_2-binding segment (residues L778-T819)*

The hTRPV1 PIP_2-interacting peptide is largely unstructured in phosphate buffer without lipids in the temperature range of 25-45 ˚C, with the exception of a short stretch of residues 811-815, where a helical structure is evidenced by the sequential d_{NN} NOEs (Figure 2A and C). In the presence of lipid bicelles (0.53 DHPC: 0.27 DMPC: 0.20 DMPG), however, the hTRPV1 peptide undergoes significant conformational change, and a great number of helical elements can be observed by dNN NOEs, especially when PIP_2 is present (Figure 2B, D, and E). Although the helical stretch of 811-815 is retained both in phosphate buffer and in lipid bicelles, the resonances have shifted significantly (Figure 2A), indicating a completely different environment experienced by the helix. Comparing the spectra of hTRPV1 in bicelles with and without PIP_2 (Figure 2B), many peaks are broadened in the hTRPV1 spectrum when PIP_2 is absent, indicating an intermediate exchange between the lipid bicelles and hTRPV1 peptide, while these peaks are clearly visible when PIP_2 is present in the sample, suggesting a reinforced interaction between the peptide and PIP_2. The broadened peaks in the absence of PIP_2 mostly come from the helical stretch of 790-794, situated in the middle of the key residues R786, K789, and R798, suggesting an essential roles of these residues in mediating PIP_2 interaction through an induced conformational change in this region.

3.3 *Molecular dynamics simulations of TRPV1 C-terminal domain*

A homology model for human TRPV1 C-terminal domain, residues G684-K839, was built from 2HE7 (FERM domain of EPB41L3) and 2R5K (Pentamer Structure of Major Capsid protein L1 of Human Papilloma Virus type 11) structures that cover the full sequence of TRPV1 C-terminal domain. The sequence alignment between TRPV1 C-terminal domain and 2HE7 and 2R5K sequences is illustrated in Figure 3. Five homology structures were generated by Modeller program [39], and the model with lowest objective function (molpdf) value and DOPE assessment score was selected for additional molecular dynamics simulations using NAMD package at 298 and 318 K, respectively. The MD models at both temperatures were properly equilibrated over 10 ns simulations, as indicated by the absence of vacuum holes between identical periodic images and small changes in volume over time of the simulated structures.

Although there is a homology model of the full length TRPV1 [27], the validity of this model is questionable. This model showed a mostly structured C-terminal domain, while the most recent experimental structure suggested a disordered domain. In addition, our experimental results do not support the C-terminal domain features reported in the model. The most discrepancy lies in the temperature sensor: our data indicates an extended secondary structure, which was confirmed by a β-strand observed in the cryo-EM structure, while the model suggested a helical structure for the same region. Our models for the TRPV1 C-terminal domain, shown in Figure 4, are, in general, consistent with the NMR experimental data, and are complementary to the cryo-EM experimental structure with limited information on the C-terminal domain. Our model structures at both temperatures show two helices at the proximal C-terminal domain, consisting of the conserved TRP box and the TRP domain that would link to the putative transmembrane segment. These two helices represent the coiled-coil domain, and are responsible for channel oligomerization. Down to the distal part of the C-terminal domain, both the temperature sensor (residues 727 -752, green) and the regulatory PIP_2-binding segment (residues 778 – 819, blue) are largely unstructured, with the exception of a short stretch of a-helix in the PIP_2-binding segment (Figure 4A). At room temperature (25 ˚C), the non-polar residues within the temperature sensor (grey, space filled) are not close to each other in three-dimensional space. At channel activating temperature (45 ˚C), the non-polar residues in the temperature sensor reorganize to form a hydrophobic core, consistent with NMR data that shows the appearance of NOE cross peaks between aromatic and valine residues. The formation of the hydrophobic core induces conformational change in the heat sensor, and extended antiparallel β-sheets are formed. At elevated temperature, additional conformational changes are also observed in the regulatory PIP_2-binding segment as the conformation becomes more extended in this region and a short stretch of helix also forms (Figure 4B). In addition, the coiled-coil

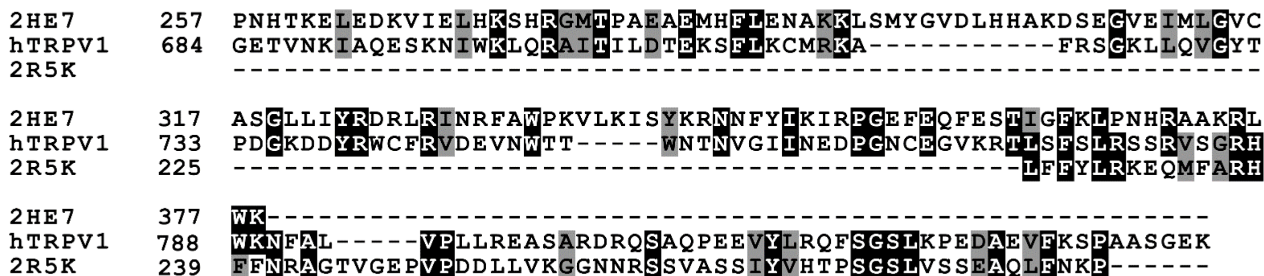

Figure 3. Sequence alignment of human TRPV1 C-terminal domain (residues 684-839) with PDB files 2HE7 and 2R5K. Identical residues are shaded black and similar residues are shaded grey. The alignment E-values are 1.3 for 2HE7 and 4.3 for 2R5K.

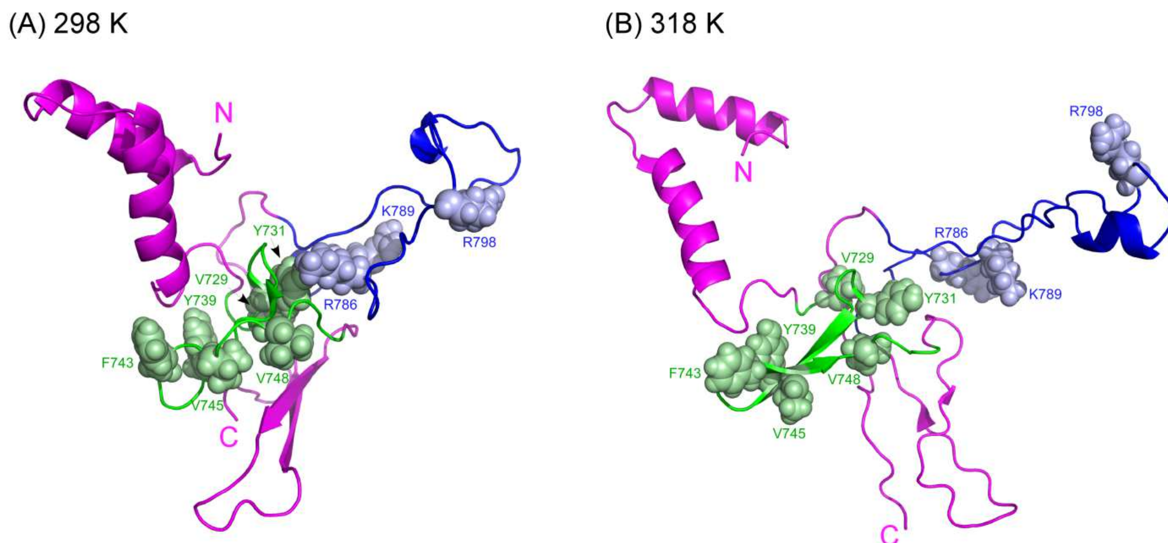

Figure 4. Computational models of TRPV1 C-terminal domain (residues 684-839) at 298 K (A) and 318 K (B). The temperature sensor (727 – 752) is colored green, and the regulatory PIP_2-binding segment (778 – 819) is blue. The key residues forming the hydrophobic core (pale green) in the temperature sensor and the residues essential for PIP_2 binding (light blue) in the regulatory segment are shown as space-filling model.

structure along the TRP box and TRP domain changes the relative orientations between the two helices, indicating a conformational change migrating from the temperature sensor into the transmembrane segment.

4. Discussion

Temperature sensing TRP channels are modular, allosterically controlled, multifunctional protein sensors that can integrate a wide variety of stimuli, including temperature, acidic pH, cell membrane voltage, and intracellular Ca^{2+} levels, respond to chemical agonists, alcohols, and endogenous cannabinoids, and induce hyperesthesia under nociceptive, inflammatory, and neuropathic conditions. The functions and responses of TRP channels are also strictly regulated by membrane-associated second messengers (such as PIP_2) and phosphorylation states. Therefore, it is important to characterize the structures and conformations of the C-terminal domain in response to temperature and PIP_2 regulation, key information currently lacking from the literature. This study reports our initial attempt in obtaining structural information and assessing conformational changes of the TRPV1 C-terminal domain at atomic details under channel activating conditions.

At room temperature, both our NMR spectra and molecular dynamics (MD) model show that most regions of the C-terminal domain, including the heat sensor and PIP_2-regulatory segments, appear unstructured with the exception of two helices around the conserved TRP box and TRP domain, a coiled-coil segment responsible for the oligomerization of TRPV1 channel, consistent with the recent cryo-EM structure of TRPV1 showing an α-helical TRP domain [14]. At channel activating temperature (45 ˚C), our NMR experimental data and the MD model demonstrated a dramatic conformational change at the heat sensor, involving the formation of various hydrophobic valine-aromatic interactions, accompanied by increasing in the β-strand components in this segment. This C-terminal β-strand component was also observed in the cryo-EM structure [14], which is an important interaction point to the N-terminal domain. This change of conformation at the heat sensor propagates throughout the C-terminal domain, including the PIP_2-interacting segment and the coiled-coil structural motif, and eventually activates the channel and opens the pore in the transmembrane segment.

Our experimental NMR results, consistent with our MD models, also show that the TRPV1 PIP_2-interacting segment is intrinsically unstructured in the temperature range of 25-45 ˚C; only a short stretch of helical structure in this segment was revealed in the MD model, which was also observed in the NMR experiments. The TRPV1 PIP_2-binding segment seemed to be able to interact with the lipid bicelles, which consists of 80% neutral lipids (DHPC and DMPC) and 20% negatively charged lipids (DMPG). The TRPV1 C-terminal domain contains several clusters of basic residues (Lys and Arg) that are positively charged under experimental conditions (pH 6.6), including a segment near the TRP box and TRP domain (residues 694-721) and the putative PIP_2-interacting segment (residues 778-819). These positively charged basic residue patches may possibly interact with the negatively charged DMPG lipid through electrostatic interactions, but such interactions are largely non-specific, as evidenced by the broadened NOE cross peaks in the presence of lipid bicelles without PIP_2 (Figure 2B). Only in the presence of 4% PIP_2 (-4 charge under experimental conditions) in the lipid bicelles, the PIP_2-interacting segment undergoes significant conformational changes, clearly adopting secondary structures, as illustrated by the shifts and appear-

ance of new NOE cross peaks (Figure 2B). We believe that the PIP_2-interacting segment is involved in a specific, electrostatic interaction with the PIP_2 molecule. This observation agrees with early studies on other PIP_2-binding peptides (such as MARCKS) that these basic membrane-bound peptides only sequester multivalent (such as PIP_2), but not monovalent (such as PG or PS) acidic lipids [41, 42].

5. Concluding Remarks

TRPV1, a multifunctional ion channel protein, is an important drug target for novel analgesics and potential modulators of the effects of substances of abuse, including alcohol and illicit drugs. However, the lack of essential structural information on TRP channels greatly limits the understanding of the channel functions and mechanisms in mediating relevant biological processes and physiological effects. Here we report our experimental NMR studies and MD simulations in revealing potential structure-activity relationship of the TRPV1 C-terminal domain. In conclusion, the C-terminal domain is largely unstructured under normal conditions (25 ˚C), with the exception of the proximal TRP box and TRP domain region, which forms a helical coiled-coil structure, and is responsible for the oligomerization of the channel. At channel activating temperature (45 ˚C), the temperature sensor (residues 727 – 752) adopts an extended secondary structure with the formation of a hydrophobic core involving several valine and aromatic residues within the sensor. The structural changes at the temperature sensor induce a substantial conformational change throughout the C-terminal domain, and transmit into the transmembrane segment to activate the channel. The PIP_2-interacting segment (residues 778 – 819) is also intrinsically unstructured, although a short stretch of α-helix was observed. This segment specifically interacts with PIP_2 (-4 charge) electrostatically through clusters of basic residues, but does not interact with monovalent lipid, such as DMPG used in this study or POPS as in the actual mammalian plasma membrane. Our study confirms the important roles of the C-terminal domain in temperature sensing and PIP_2-regulation of the TRPV1 channel functions, and provides structural insights into the mechanisms of the C-terminal domain in mediating these stimuli. Although a high-resolution cryo-EM structure of a minimum TRPV1 construct is now available, this structure lacked the regulatory PIP_2-binding segment of the C-terminal domain, and besides the α-helix of the TRP domain and a β-strand within the temperature sensor, limited information was available for the functionally critical C-terminal domain in this experimental structure. Our models of the full C-terminal domain, complementary to the experimental structure, therefore, provide key information in understanding the channel functions.

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