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In this issue

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The Canadian Society for Clinical Investigation
La Société Canadienne de Recherches Cliniques

CSCI SCRC

About CSCI

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To promote clinical and basic research in the field of human health throughout Canada, to lobby for adequate research funding at the federal, regional and local levels, and to support Canadian researchers in their endeavours and at all stages of their careers.

The Society still fulfills its original mandate today. It has evolved, however, to include the active promotion of clinical science and lobbying for support of basic and applied biomedical research from the federal and provincial governments. CSCI members represent researchers across Canada who are studying issues of disease and health care across the spectrum, from basic research to issues of health care delivery.

ORIGINS

The Canadian Society for Clinical Investigation (CSCI) was founded in 1951 and its original purpose was to provide a forum for the exchange of scientific information. It was envisaged as a "travel club for those interested in clinical investigation in Canada". As detailed by J.S.L. Browne, one of the four founding members of the CSCI, the idea was for it to be a very informal organization and not a society.

Its first meeting was attended by 44 people and was an outstanding success. Over the next several years, discussion continued as to the proposed nature, structure and organization of a society for Canadian clinical investigators. These discussions culminated in the formation of the CSCI in 1959. Its first meeting was held in Vancouver that year and the meetings have continued to grow in size and are now held conjointly with the annual meeting of the Royal College of Physicians and Surgeons of Canada.

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EDITORIAL PAGE

Health Issues Associated with Societal Evolution

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Society has evolved from generalized hunter gathers to specialized service providers. Humans used to have relatively undifferentiated lives that involved sustained physical activity and diets consisting of relatively unprocessed foods. Physical fitness and nutrition were essential to longevity and death was typically from starvation, infections, environmental exposure and trauma. Since the first modern steam engine in 1765 that heralded the industrial revolution[1], mechanization and power driven automation have provided the means to mass produce the necessities of life and comforts in previously inconceivable quantities. The requirement for specialized knowledge and skills to run automated equipment has resulted in most having sedentary service careers. The food industry mass produces rapid sources of food that are calorically dense. Advances in public health and in medicine have resulted in dramatic increases in longevity. The future promises greater advances in automation and medicine. Despite the huge benefits, there are increasing concerns that societal evolution is resulting in unheralded new public health problems that are largely a result of the 'advances' in our society. A substantial concern is that new understanding and approaches to public health are required but not currently occurring. This commentary addresses some of the new health issues associated with decreased physical activity, changes in dietary habits and increased body mass associated with our societal evolution.

Great advances of the human race include electricity, powered machinery and computers. There are few vocations that have not had huge increases in efficiency through these advances. The result however is that the physical effort required in most vocations has been greatly reduced. Further, the advances have dramatically affected personal lives outside work. The invention of the automobile and electrical equipment has improved human efficiency tremendously. Many household tasks that once involved substantial effort and time are now rapidly performed with relatively little effort. The improved efficiency has resulted in increases in recreational time. However, technological advances have also resulted in increases in sedentary recreational time spent watching television and playing computerized games. Perhaps the greatest impact on recreational time has been in children where sedentary recreation is typically measured in hours per day.

The food industry has also been affected by societal evolution. To increase efficiency, most foods are mass produced and altered for preservation, rapid preparation and consumption. Processing conserves or concentrates the caloric content of food but has not maintained the balance of nutrients (especially non-caloric nutrients). Food has become increasingly available and costs have been reduced particularly for large portions or bulk food.

With these advances, new health concerns have emerged. Sedentary vocations and recreational time coupled with large quantities of calorically dense foods has resulted in alarming increases in body mass. Physical inactivity, a refined westernized diet and increased body mass cause high blood pressure, diabe-

tes, and dyslipidemia. Combined, these factors result in atherosclerotic cardiovascular disease and certain cancers, the leading causes of mortality in industrialized countries and rapidly becoming leadings causes of death in developing countries.

Although our societal evolution has resulted in improved health and longevity, there are worrisome trends with increased obesity and diabetes at younger ages, with future implications for cardiovascular disease. Atherosclerotic cardiovascular disease is a global epidemic that threatens the improvements in health brought about by societal evolution. Technological advances in medicine are increasingly expensive to apply to large proportions of the population. The wealthy not only maintain healthier lifestyles, but are also able to afford expensive individualized interventions that are designed to circumvent unhealthy lifestyles. The result is that there will likely be enlarging gulfs in health between those with and those without substantial financial resources.

A fundamental issue is to ensure that public health continues to evolve to benefit from continued technological advances. Responsibility for ensuring healthy evolution rests with corporations, individuals, not-for-profit sectors and governments. Major advances have been driven by a competitive market place. In this environment, many companies have increased in size and resources such that the gross income of some companies exceeds that of small to mid-sized countries. In general, these corporations drive improvements in efficiency and innovation that are now associated with the new public health epidemic. However, the prime responsibility of companies is to their owners (shareholders). Although many corporations are starting to recognize their potential negative influence on public health, it is unlikely that corporations will directly improve public health without economic incentive. Individuals can influence governments and corporations and are responsible for making healthy choices. Unfortunately, changes in our society have made healthy choices difficult. Unprocessed fresh food in recommended portions is not as accessible as processed food and is more expensive. Incorporating physical activity into a daily routine is difficult for both children and adults.

Perhaps, far sighted politicians could guide corporations and individuals through regulation and legislation. Just as child labour issues and pollution that plagued the early industrial revolution were dealt with by governments it is time to act during the current health system crisis. A series of incentives and deterrents are needed to make healthy choices a routine way of life in our communities. Regular activity and healthy balanced diets should be made the default choice, whereas unhealthy choices should be made expensive and inconvenient.

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ORIGINAL ARTICLE

Epidemiology and Outcome of Bacterial Meningitis in Canadian children: 1998-1999

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Abstract

Background: The introduction of *Hemophilus influenzae* type b (Hib) conjugate vaccine as part of the routine childhood vaccination schedule in Canada has resulted in a dramatic reduction in the cases of Hib meningitis. We describe the epidemiology and outcome of bacterial meningitis in Canadian children six years after the introduction of Hib conjugate vaccine and prior to the introduction of the conjugate *Streptococcus pneumoniae* vaccine.

Methods: A retrospective chart review from January 1998 to December 1999 of children with meningitis identified at eight Canadian tertiary care children's hospitals belonging to the PICNIC network.

Results: Bacterial meningitis was documented in 104 (11%) of 970 children presenting with meningitis. The most common isolated organisms were: *Streptococcus pneumoniae* (54%), group B streptococci (13%), and *Neisseria meningitidis (*11%). The mean age was 2.2 ± 3.5 yr. Forty seven percent of the children required admission to Intensive Care Unit (ICU), and 19% required artificial ventilation. Sequelae were documented among 32 children (31%) prior to discharge and there were 6 (5.6%) deaths attributable to meningitis and sepsis.

Conclusions: Bacterial meningitis is an important cause of morbidity in Canadian children with *S. pneumoniae* replacing *H. influenzae* as the leading potentially vaccine preventable cause. Despite proper initiation of antimicrobial therapy, meningitis results in great morbidity and mortality in children in Canada.

Bacterial meningitis is an important cause of morbidity and mortality in children despite the advent of highly effective, aggressive medical management and early institution of intravenous antibiotics. In United States and before the institution of the conjugate pneumococcal vaccine, there were close to 6000 new cases of bacterial meningitis each year, half of which occurred in children less than 18 years of age.[1] There was a dramatic decline in the incidence of *Haemophilus influenzae* type b (Hib) meningitis following the introduction of conjugate Hib conjugate vaccine.[1-3] Furthermore, the introduction of the heptavalent *Streptococcus pneumoniae* conjugate vaccine (PCV7) in June 2000 had dramatically reduced the incidence of pneumococcal invasive disease including meningitis in up to 66% of children equal or less than 24 months of age in 2002 in the United States.[4.]

In Canada, two studies have reported on the decline of Hib meningitis and clusters of meningococcal disease [5,6] , but none described the epidemiology of bacterial meningitis in Canadian children after the introduction of Hib conjugate vaccine. Therefore, we conducted this comprehensive national retrospec-

tive study to describe the epidemiology and outcomes of patients with bacterial meningitis in Canadian children after six years of the introduction of conjugated Hib vaccine and prior to the introduction of conjugate pneumococcal vaccine in Canadian provinces.

Methods

Patients: All children with meningitis were identified by retrospective chart review at eight Canadian Children Hospitals between January 1, 1998 and December 31, 1999. All patients with *International Classification of Diseases, Ninth Revision* (ICD-9) codes that may represent meningitis were examined for eligibility. Participating hospitals are members of the Pediatric Investigators Collaborative Network on Infections in Canada (PICNIC), a collaborative network of pediatric infectious disease specialists who conduct studies at the university affiliated pediatric centers in Canada (URL: http://www.picnic-canada.org). The local ethics review boards of all participating sites reviewed and approved the study before it started.

Cases were classified as bacterial meningitis if they met one of the following diagnostic criteria: (a) Cerebro Spinal Fluid (CSF) pleocytosis, defined as WBC ≥25/mm^3 for a term neonate and ≥5/mm^3 for a child plus (b) an evidence of CSF bacterial infection documented either by a positive bacterial culture, a positive gram stain or a positive latex agglutination test. Patients were excluded if (a) had underlying central nervous system tumor, (b) had central ventricular shunts or had recent brain surgery (c) CSF grew coagulase negative staphylococci (d) CSF had a mixed growth or (e) premature baby who was hospitalized in neonatal intensive care unit (NICU) more than a week at the time of diagnosis of meningitis.

Data collection: A standardized data collection sheet was used among all participating centers and was completed for patients enrolled including demographic data, clinical data, laboratory data, management in the hospital and clinical outcome.

Statistics: The data were managed at the Child Health Research Unit, Alberta Children's hospital. All data were entered onto Microsoft ® Access 97 database. This study was mainly descriptive in nature, so descriptive statistics were used.

Results

Demographics and patient characteristics: Nine hundred and seventy patients with the diagnosis of meningitis were identified during the study period. One hundred and four (11%) patients met the inclusion criteria for bacterial meningitis. The diagnosis was made by CSF culture in 94 cases (90%), CSF antigen detection in 4 cases (4%) and CSF pleocytosis plus gram stain in 6 cases (6%). Fifty-four patients (58%) were male. The median age was 1 year (range 1 day – 17 yr) with a mean of 2.2 ± 3.5 yr. Twelve (11%) of the cases occurred in neonates, 64 cases (62%) in children 1- 24 months of age and 28 cases (27%) in children more than two years of age. Nine children (9.6%) had underlying chronic medical conditions: two with immunodeficiency, three were receiving chronic steroid therapy and four had bronchial asthma. Twenty-four patients (26%) had received antibiotics a week prior to presentation with a median of two days.

Bacterial etiologies: The agent most commonly associated with bacterial meningitis was *Streptococcus*

TABLE 1: Organisms identified in 104 Pediatric Cases of Bacterial Meningitis in Canada 1998-1999

Organism	*No. of cases*	*Percentage of total (%)*
Streptococcus pneumoniae	58	56
Group B streptococci	14	13
Neisseria meningitidis	11	11
Haemophilus influenzae Type B	8	8
E.coli	5	5
#Other gram negative organisms	3	3
Haemophilus influenzae Type A	3	3
*Other gram positive organisms	2	2

#: include *Acinetobacter spp*, *Enterobacter spp.*
*: include: *Enterococci* and *Staphylococcus aureus*

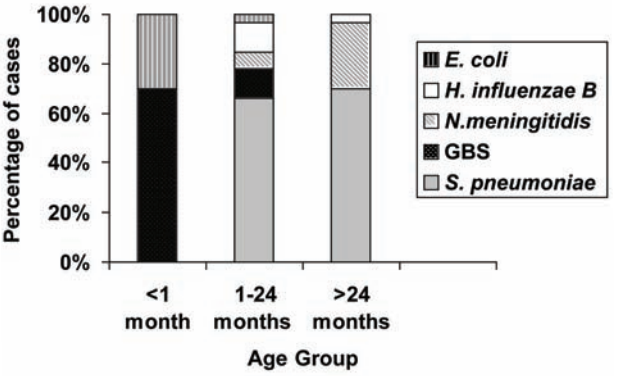

FIGURE 1: Bacterial Agents of Meningitis According To Age Group.

TABLE 2: Intensive Care Unit Admission And Ventilation of Patients With Bacterial Meningitis By Pathogen

Bacterial pathogen	*ICU admission (%)*	*Ventilation (%)*
Streptococcus pneumoniae	29 / 58 (50.0)	12 (20.7)
Group B Streptococci	10 /14 (71.4)	5 (35.7)
Neisseria meningitidis	6/11 (54.5)	2 (18.2)
Haemophilus influenzae Type B	2/8 (25.0)	1(12.5)
Haemophilus influenzae Type A	2/3 (66.7)	-
Overall	49/103 (47.6)	20/103 (19.4)

pneumoniae, followed by Group B streptococcus and *Neisseria meningitides* (Table 1).

The predominant agents associated with bacterial meningitis varied according to the age group (Figure 1). Group B streptococcus was the main organism responsible for bacterial meningitis during the neonatal period accounting for 58% of the cases. *S. pneumoniae* was the leading bacterial agent for bacterial meningitis in children beyond neonatal period.

Management and outcomes: All patients received antibiotics on admission. The most common given antibiotics were a combination of third generation cephalosporin (cefotaxime or ceftriaxone) and vancomycin in 82% of children older than three months of age and a combination of ampicillin and cefotaxime in 82% for children younger than three months of age. Only 26 (25%) patients received dexamethasone. Forty-nine patients (47%) were admitted to the Intensive Care unit (ICU) and 20 (19%) required mechanical ventilation for a mean duration of 3.5± 3.5 days. Table 2 lists the main bacterial pathogen requiring ICU admission and ventilation.

Six patients (5.8%) died. All deaths occurred in children in the 1- 24 months age group. Five patients had *S. pneumoniae* and one had Group B streptococcus meningitis. Thirty two patients had their hospitalization course complicated with 39 events. The most common complications were seizures and hearing loss. *S. pneumoniae* was the most common bacteria associated with complication responsible for 66 % of all reported complications (Table3).

Discussion

This comprehensive retrospective Canadian collaborative study of bacterial meningitis at eight tertiary Children's Hospitals over two years examined etiologic agents and epidemiological features of affected children and their outcome. The most significant finding was that after the eradication of Hib, *S. pneumoniae* was the leading bacterial agent resulting in more than half of the cases of meningitis. The other finding was that despite the advances in antimicrobial therapy, meningitis remains a disease with great impact on young children resulting in acute neurological sequelae in 31% and considerable hospital morbidity in the form of intensive care admission in half of the cases and the need for mechanical ventilation in a substantial number of them.

In Canada, bacterial meningitis remains a disease predominantly of younger children with 73% of the cases in children in the first two years of age similar to other reports.[7,8] Routine Hib conjugate vaccination had resulted in the decline of the rate of invasive Hib disease in Canada from 2.56 /100 000 in 1988 to 0.09/100 000 in 2000.[9] Meningitis caused by other bacteria remained a disease of the first two years of life similar to other reports.[1,2]

S. pneumoniae is the most common bacterial meningitis agent for all ages beyond neonatal period following the introduction of Hib conjugate vaccine in all Canadian provinces in 1992.[5,10] *N. meningitidis* is the second commonest causative agent after *S. pneumoniae* in children older than two years of age. This organism dominance is different to what is expected given the endemicity of meningococcal disease and

TABLE 3: Ceomplications of Bacterial Meningitis Reported Among 104 Patients And Distribution In Relation To Bacterial Pathogen (39 complication events in 32 patients)

Sequelae	*S. pneumoniae*	*GBS*	*N. meningitidis*	*H. flu B*	*H. flu A*	*Total (%)*
Hearing impairment	10	-	1	1	-	12 (11.5)
Seizures	6	2	-	1	2	11(10.5)
Motor deficit	4	-	-	-	1	5 (4.8)
Cranial nerve palsy	4	-	1	-	-	5(4.8)
*Intracranial complications	1	1		1	-	3 (2.8)
**Non neurological	1		1	1	-	3 (2.8)
Total	26	3	3	4	3	

* includes: intracerebral hemorrhage (1) subdural effusion (1), hydrocephalus (1).
** includes: arthritis (2), pneumothorax (1)

to what was described by Schuchat in United States where *N. meningitidis* was found to be the principle cause of bacterial meningitis in children 2-18 yr.[1] This can be explained by the low rates of meningococcal disease during the study period as the last major meningococcal disease epidemic occurred in 1940-1943 (serogroup A), with an incidence of 13 per 100,000 population per year. Since then, the overall incidence of disease has remained <= 2 per 100,000 per year (range 0.5 to 2.1). There have been sporadic localized outbreaks and periods of elevated activity (serogroup C) during 1989-1993 and 2000-present.[11] We have not examined the serotypes of *Neisseria* since this is beyond the scope of this study.

An interesting finding in this study was that, during 1999, Hib remained an important cause of meningitis in children in the first two years of life accounting for 8% of the cases. This can be attributed to no vaccination or failure of the vaccine.[10] A subsequent report from the Immunization Monitoring Program, Active (IMPACT) has shown a decrease in the number of Hib meningitis in the year 2000 to only two cases.[13] The three cases of *H. influenzae* type A in our cohort support the few published reports that during the Hib conjugate vaccine era other serotypes of *Haemophilus* have emerged as causes of *H. influenzae* meningitis in fully vaccinated children.[13-15]

Despite advances in medical care, life support, and appropriate antibiotic treatment, the outcome was characterized by serious morbidity. Thirty- eight percent of children had short term sequelae. Others have reported a similar rate but as long- term sequelae.[16] Although this study was not designed to look at long-term sequelae, it highlights the morbidity and the impact on families and the health care systems. The case fatality rate for this cohort with bacterial meningitis was in keeping with the published report of 2-17%. [7, 17]

The data presented here is valuable to assess the burden of bacterial meningitis in Canada before the implementation of conjugate pneumococcal vaccine as part of the routine childhood immunization schedule. The seven *S. pneumoniae* serotypes (14, 6B, 19F, 18C, 4, 23F, and 9V) account for more than 80% of invasive isolates from children < 5 years of age which are the serotypes included in the vaccine currently used.[18]

Despite that this study provides a baseline for future evaluation of the epidemiology and outcome of childhood bacterial meningitis in Canada, certain limitations should be noted. The number of cases in this study reflects all tertiary centres and did not include community hospitals where patients with simple uncomplicated meningitis have been admitted. This might have accounted for the increase in the morbidity and mortality of this cohort. Also, there might be an underestimate of the total number of bacterial meningitis cases as patients with clinical meningitis without lumbar puncture were not included in this study. However, we believe this is highly unlikely for bacterial meningitis. Furthermore, this study was not designed to look at the long tem outcome of patients with bacterial meningitis, which reflects the true burden of this infection. Another limitation of this study was that we did not examine the antimicrobial susceptibility of the organisms identified and hence we were not able to associate if adverse events were due to resistant organisms.

In conclusion, the epidemiology of bacterial meningitis in Canada had changed similar to other countries where Hib conjugate vaccine is routinely used with *S. pneumoniae* as the leading organism. Significant patients were severely ill requiring ICU admission and a great number of them had neurological morbidity. This study provides a baseline to evaluate future change in the epidemiology and outcome of meningitis after the routine use of the conjugate pneumococcal vaccine for immunization in all Canadian provinces.

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ORIGINAL ARTICLE

Identification of Protein Biomarkers in Dupuytren's Contracture using Surface Enhanced Laser Desorption Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF-MS)

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Abstract

Background: To study the protein expression profiles associated with Dupuytren's contracture (DC) to identify potential disease protein biomarkers (PBM) using a proteomic technology - Surface Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF-MS).
Methods: Normal and disease palmar fascia from DC patients were analyzed using Ciphergen's SELDI-TOF-MS Protein Biological System II (PBSII) ProteinChip® reader. Analysis of the resulting SELDI-TOF spectra was carried out using the peak cluster analysis program (BioMarker Wizard, Ciphergen). Common peak clusters were then filtered using a bootstrap algorithm called SAM (Significant Analysis of Microarrays) for increased fidelity in our analysis.
Results: Several differentially expressed low molecular weight (<20 kDa) tissue proteins were identified. Spectra generated using both $ZipTip_{C18}$ aided Au array and WCX2 array based SELDI-TOF-MS were reproducible, with an average peak cluster mass standard deviation for both methods of $<1.74 \times 10^{-4}$. Initial peak cluster analysis of the SELDI spectra identified both up-(14) and down-(3)regulated proteins associated with DC. Further analysis of the peak cluster data using the bootstrap algorithm SAM identified three disease-associated protein features (4600.8 Da, 10254.5 Da, and 11405.1 Da) that were elevated (5.45, 11.7, and 4.28 fold, respectively, with a 0% median false discovery rate).
Conclusion: SELDI-TOF-MS identified three potential low molecular weight tissue protein markers ($p4.6^{DC}$, $p10^{DC}$, $p11.7^{DC}$) for DC. The ability of SELDI-TOF-MS to resolve low molecular weight proteins suggests that the method may provide a means of deciphering the biomarker-rich low molecular weight region of the human proteome. Application of such novel technology may help clinicians to focus on specific molecular abnormalities in diseases with no known molecular pathogenesis, and uncover therapeutic and/or diagnostic targets.

Dupuytren's contracture (DC) is a benign and often familial fibro-proliferative disorder of the hand.[1-3] Despite its long clinical history and remarkably high incidence among Caucasian populations, the genetic etiology of DC is still unknown. This lack of basic

understanding of the molecular patho-physiology of DC has resulted in an absence of significant treatment advances over the last decennia and surgical resection remains the mainstay of therapy. To rationally explore alternative treatment modalities for this disease, it would be necessary to identify potential therapeutic targets, as well as diagnostic markers that may indicate disease progression of DC.

The recent advances in DNA informatics (e.g. the Human Genome Project) offer a unique opportunity to study human disease in an innovative fashion. In addition, the availability of new technology such as micro-fabricated DNA microarrays and genome-wide single-nucleotide polymorphisms (SNPs) markers, have now allowed researchers to monitor the expression levels of thousands of genes simultaneously or to explore genotype-phenotype relationships.[4] For DC, the application of these types of technologies has only very recently been reported. Pan and colleagues used a small gene microarray to look at changes in gene expression associated with DC.[5] Several genes were shown to be consistently elevated in DC patients, however, the limited sample size and lack of meaningful statistical quantitative information about the levels of expression of these genes have been raised as criticism of this work. Undoubtedly, this is related to the relatively high cost of using this type of DNA array technology. In contrast, Bayat and colleagues have carried out a number of genetic association studies by examining common SNPs for several disease candidate genes that are components of the transforming growth factor-β (TGF-β) signalling pathway (i.e. TGF-β1, TGF-β2, TGF-β receptors I, II, III, and Zf9) [6-8]. Using a PCR-based restriction fragment length polymorphism (PCR-RFLP) method, this group found differences in genotype and allele frequencies for Zf9, a transcription factor known to enhance the fibrogenic potential of TGF-β1.[9, 10] Although there is great promise in using SNPs genotyping to help pinpoint disease-associated genes, there are many confounding factors that can cause false-positive genetic associations (e.g. population artefacts, non-functional SNPs, etc.).

A relatively novel alternative to DNA-based informatics is the use of proteomic-based technologies to study human health and disease, particularly mass spectrometry (MS). Advances in MS instrumentation, sample preparation and ionization techniques, and refinements in MS algorithms have now made it possible to analyze complex protein mixtures (e.g. serum) directly, on a large scale, and potentially in a high-throughput manner. Of all the recently developed MS techniques, Surface Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF-MS) has perhaps garnered the most attention with respect to disease biomarker discovery (Figure 1). SELDI-TOF-MS is a highly sensitive form of Matrix-Assisted LDI (MALDI)-TOF technique, which uses special chromatographic-like probe surfaces (Protein Chip® arrays) to capture different classes of proteins.[11, 12] Unlike the inert metal probe surfaces used in MALDI-TOF, the SELDI Protein Chip® arrays provide specialized protein-

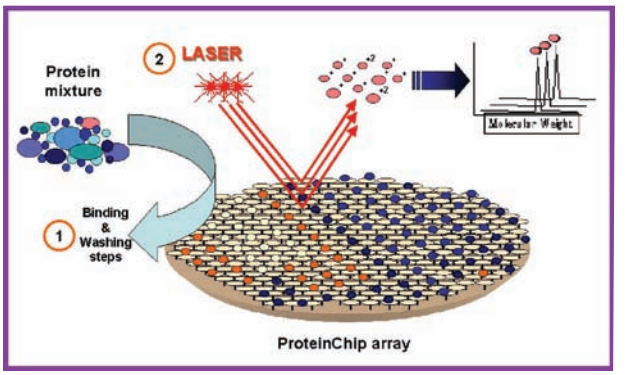


FIGURE 1 - Surface Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry: General steps involved in the ProteinChip® based SELDI-TOF-MS. Unlike traditional MALDI-TOF-MS, which uses an inert metal probe surface to present the sample for MS-TOF analysis, ProteinChip® arrays play an active role in the TOF-MS process and permit direct analysis of crude complex protein mixtures (e.g. serum). **(1)** Bbriefly, small aliquots (1μl) of equivalent protein are spotted onto the active regions of 8-spot ProteinChip® arrays. Binding conditions are varied to reduce background and possibly facilitate sequential 'on-chip' protein purification. Following protein binding each spot or the entire array is washed using HPLC grade H_20 or more stringent buffers (e.g. PBS + 0.2M NaCl + 0.1%Tween). A laser energy-absorbing-matrix (EAM, e.g. 100% saturated solution of cyano-4-hydroxy cinnamic acid, or CHCA) is then applied to each spot and allowed to dry thoroughly. **(2)**The array is then placed in the SELDI ProteinChip® Biology System II (PBS II) reader for scanning using an automated data collection protocol. The PBS II system is configured such that the only variable determining its TOF within the drift tube is its mass-per-unit-charge (m/z). Thus, smaller and/or more highly charged ions will reach the detector first, with the abundance of any given ion being proportional to its peak amplitude or area.

binding surfaces (e.g. quaternary amine functionality) that are capable of capturing proteins with complementary physiochemical properties (e.g. anionic proteins). SELDI-TOF-MS is, therefore, particularly well-suited for clinical proteomic studies since it can rapidly detect disease-specific protein profiles in a largely unbiased manner without prior knowledge of the underlying genetic lesions, while using only small quantities of patient material.[13-15] While this method preferentially detects low molecular weight (<30kDa) proteins, more comprehensive proteomic profiling is possible when simple, high-throughput sample fractionation techniques are used in combination with SELDI-TOF-MS.[16] Because of these reasons, we have employed this TOF-MS technique to identify changes in protein expression associated with DC.

METHODS

Clinical specimen and primary cultures: DC patient specimens (normal and disease palmar fascia) were collected in compliance with the University Human Research Ethics Board. Areas of diseased fascia (disease) and adjacent, uninvolved normal palmar fascia (control) were collected from DC patients undergoing surgical resection of diseased palmar fascia tissue. Portions of the clinical specimens were stored at -80°C for protein extraction.

Preparation of protein extracts for SELDI-TOF analysis: Protein extracts were prepared using a commercially available tissue-protein extraction reagent T-PER (Pierce, Rockford, IL, USA), supplemented with Sigma protease inhibitor cocktail (2mM AEBSF, 1mM EDTA, 130µm Gestating, 14µm E-64, 1µm eupeptic, 0.3µm apportioning) + 1mM PMSF (paramethylsulfonyl fluoride, Sigma), and general phosphatase inhibitors (1mM Na_3VO_4, 1mM NaF, Sigma, St. Louis, MO, USA). Tissue samples were homogenized (Ultra-Turrax T8, IKA Labortechnik, Staufen, Germany) using short bursts (high setting) under ice to avoid mechanical heating of the samples. The resulting sample homogenates were briefly centrifuged (15min, 4°C, 12,000 x g) to remove insoluble debris. The resulting supernatant was then assayed for total protein content using a commercially available BCA protein assay kit (Pierce, Rockford IL, USA).

SELDI-TOF-MS analysis using WCX2 ProteinChip® arrays: For SELDI-TOF analysis using weak cation exchanger (WCX2) ProteinChip® arrays, equivalent protein (5µg) was suspended in a final 20µl volume containing 12.5mM sodium acetate (pH 5.0) and 0.1% triton X100. HPLC grade H_20 (Sigma) was used for all sample preparations. Prior to applying the samples onto the WCX2 arrays, the active spots of the array were pre-activated in accordance with the manufacturer's instructions. Briefly, the entire WCX2 chips were washed for five minutes in 0.01N HCl, followed by two quick washes in HPLC grade H_2O. A hydrophobic PAP pen was then used to create a containment barrier around each active spot of the WCX2 arrays. Once the hydrophobic barrier was dry the active spots of the array were washed 2x (10min washes, RT) with binding buffer (12.5mM sodium acetate, pH 5.0 + 0.1% triton X100). The samples (5µl containing 5µg total protein) were then applied to each pre-activated spot of the array and allowed to bind (20 min., 22°C, humidified chamber) to the array. Following the binding step, the entire WCX2 array was then washed (2x) with binding buffer (10 min, 22°C, rotator) and then once with HPLC H_2O. After briefly drying the arrays, 0.5µl of a saturated solution of 4-Hydroxy-3,5-dimethoxy-cinnamic acid (sinapinic acid or SPA, Sigma) dissolved in 50% (v/v) acetonitrile (ACN) and 0.5% (v/v) trifluoroacetic acid (TFA), was applied 2x to each of the active spots of the array and allowed to thoroughly dry. The WCX2 array was then placed in the SELDI ProteinChip® Biology System II reader (PBS II) for scanning using an automated data collection protocol, as we have previously described.[13, 17, 18]

The PBS II reader is a linear TOF-MS equipped with a 337nm nitrogen laser. It was operated in positive-ion mode with a uniform static electric field of +20kV. The resulting spectra were averaged from at least 65 collected transient laser shots that scan ~66% of the target area in linear sweeps (5 shots/region, 13 regions per spot). Sequential laser scans were carried out by incremental increasing the laser intensity (LI) with each subsequent scan to optimize data collection for both low and high molecular weight proteins (LI settings of 175-295, with a LI of 185 equivalent to ~5µJoules).

$ZipTip_{C18}$ aided SELDI-TOF-MS analysis using Au ProteinChip® arrays: SELDI-TOF-MS analysis of $ZipTip_{C18}$ prepared surgical specimens was carried out using gold (Au) probe surfaces (Figure 2). $ZipTip_{C18}$ sample preparation, which concentrates and desalts the protein mixture, was carried according to the manufacturers instructions (Millipore Corporation, Billerica, MA USA). Briefly, 5µg protein (disease, or control tissue extract) was suspended in a final volume of 20µl HPLC grade H_2O (Sigma) containing 0.5% TFA (v/v). The acidic conditions (pH < 4) ensure

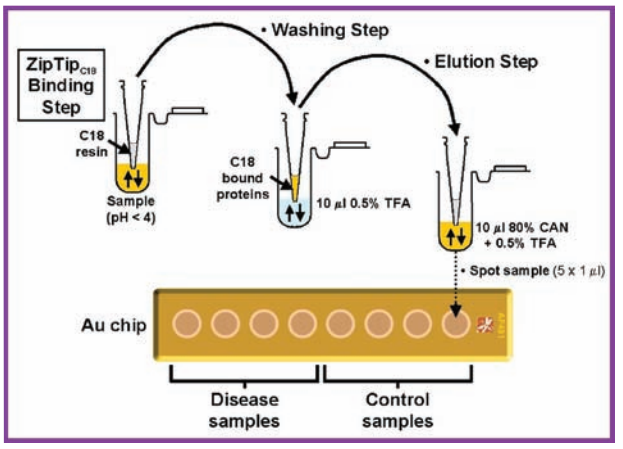


FIGURE 2 - SELDI-TOF-MS analysis using ZipTip$_{C18}$ and Au arrays: DC samples were processed using ZipTip$_{C18}$ prior to performing matrix assisted LDI-TOF-MS analysis using a simple gold (Au) chip probe. Depicted above is a single elution step approach to using the ZipTip$_{C18}$ pipette tips, which contains 0.6 µl of C18 reversed-phase media impregnated at its end. After ZipTip$_{C18}$ wetting (50% acetonitrile (ACN)) and equilibrating (0.5% TFA), sample binding was carried out by pipetting the sample up and down (10 cycles) through the fixed volume of equilibrated C18 resin. After binding the ZipTip$_{C18}$ was immediately washed (0.1% TFA, 5 pipette cycles) and the bound proteins eluted with a fixed volume (10µl) of 80% ACN + 0.5% TFA. Eluted fractions were then spotted (5 x 1µl) onto the Au probe surface and allowed to dry. Matrix (100% CHCA) was then applied to the sample spots on the Au probe surface and allowed to dry thoroughly. The Au probe was then placed in the SELDI ProteinChip® Biology System II (PBS II) reader for scanning using an automated data collection protocol.

maximum binding to the reversed-phase (0.6 µl bed volume of C_{18} media) ZipTip pipette tips. Following wetting and equilibriating of the ZipTip$_{C18}$ pippette tips with 50% (v/v) ACN and 0.1% (v/v) TFA, respectively, protein binding was carried out. Briefly, the protein mixture was cycled (aspirating and dispensing) 10x through the fixed, reverse phase bed volume of the ZipTip$_{C18}$ pippette tips. Following binding, the protein loaded ZipTip$_{C18}$ pipette tips were washed (three pipette cycles) with 0.1% (v/v) TFA. Elution of the ZipTip$_{C18}$ bound proteins was then carried out by cycling (10x) a single volume (15µl) of elution buffer (80% (v/v) ACN + 0.1% (v/v) TFA) up and down through the ZipTip$_{C18}$. The elution fraction (5 x 1µl volumes) was then applied to each spot of an Au ProteinChip® array and allowed to thoroughly dry at RT. Finally, 0.5µl of a saturated solution of α-cyano-4-hydroxy-cinnamic acid (CHCA, Sigma) in 50% (v/v) ACN + 0.5% (v/v) TFA was applied (2x) to each spot and allowing to thoroughly dry. The Au arrays were then placed in the SELDI ProteinChip® Biology System II reader (PBS II) for scanning using an automated data collection protocol as described above.

Biomarker Peak Cluster Analysis: SELDI ProteinChip® spectra were analyzed using the PBS II ProteinChip® software (v3.1.1). Briefly, calibrated spectra were initially normalized to total ion current (all peaks > 1500Da) to compensate for small variations in sample concentrations per spot or any m/z shifts in the spectrum. Peak cluster analysis was then performed using the Biomarker Wizard software (v3.1.1), which statistically evaluates clusters of peaks of similar MW from across sample groups of spectra. The initial peak detection scan was carried out using a signal-to-noise (S/N) ratio of ≥5. A second peak detection scan was then performed using a S/N ≥ 2 to ensure peak statistical analysis across all groups.

SAM Protein Filtering: Further statistical analysis of the peak cluster data sets was carried out using the bootstrap algorithm SAM (Significance Analysis of Microarrays, see: http://www-stat.stanford.edu/~tibs/SAM).[19] Although SAM was originally developed for Microarray gene expression analysis it has also been effectively used to filter SELDI-TOF data .[16] In this study, SAM was used to compare normalized peak cluster intensities between disease and control fascia groups (ProteinChip® BioMarker Wizard program, ProteinChip® Software v3.1.1, Ciphergen Bio systems Inc.). Data were exported to an Excel spreadsheet file using an Excel plug-in feature and labelled in accordance with SAM guidelines, with normalized peak cluster intensities values of the control group (n = 9) defined as group "1" and the disease group (n = 16) defined as group "2". Data analysis parameters included "two classes unpaired data" and 5000 permutations. An operator controlled (i.e. sliding) significance difference threshold, or delta value (Δ), permits the user to define the false discovery rate (FDR) (i.e. # falsely called proteins / # differentially expressed proteins) of the analysis. For each SAM analysis a table of Δ values was generated, which contained their associated FDR (median and 90th percentile).

RESULTS

Identification of DC protein biomarkers (PBM) using ZipTipC18 aided Au array SELDI-TOF-MS: Peak cluster analysis (Biomarker Wizard program,

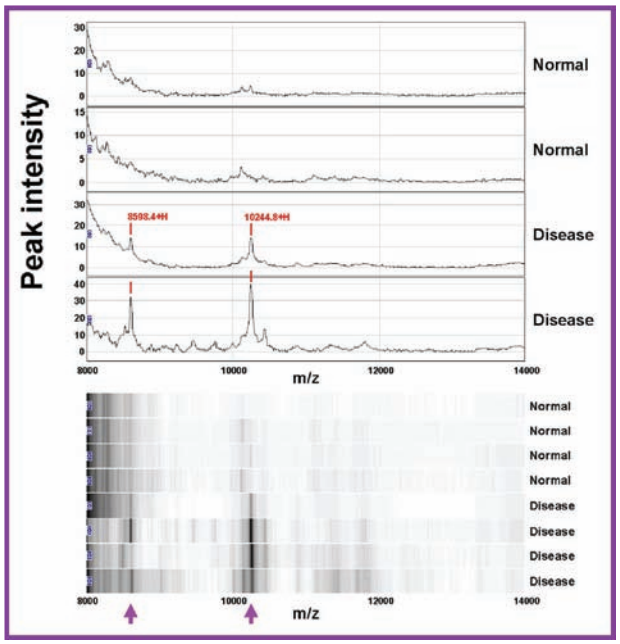
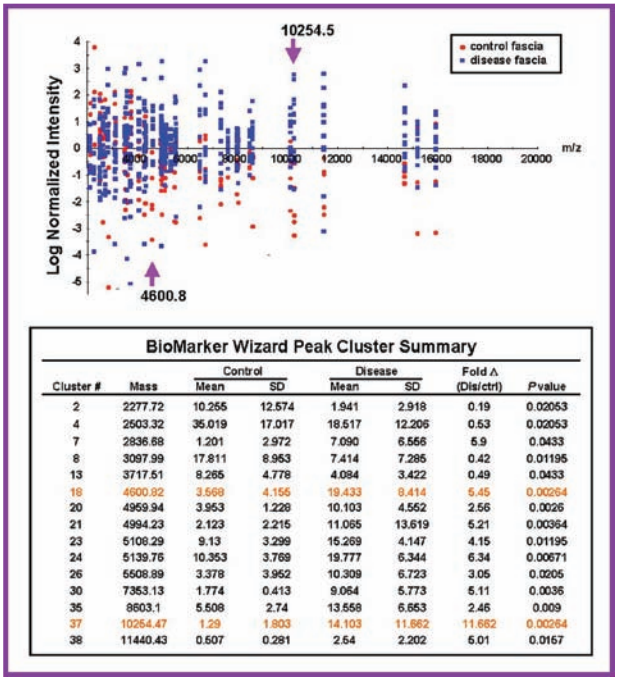


FIGURE 3 - ZipTip$_{C18}$ aided Au array SELDI-TOF-MS analysis of DC: Protein (5µg) extracted from normal and disease fascia was diluted in TFA (0.5%) to a final volume of 20µl. The protein samples were then allowed to bind to ZipTip$_{C18}$ pipette tips as described above (see Figure 2). ZipTip$_{C18}$ eluted fractions were spotted (5 x 1µl) onto Au chip probes and allowed to dry. EAM (2 x 1µl of CHCA) was applied to the gold chip spots, air dried and then placed in the PBS II reader for scanning using an automated collection protocol. The spectral data corresponding to peak intensity versus m/z were generated using 65 laser shots per laser intensity setting. Shown above are representative spectra (peak intensity, upper panel, and pseudo-gel view, lower panel) of 4 control and 4 disease patients. *Arrows show two protein peaks that were identified as significantly elevated in the disease tissue using the BioMarker Wizard peak cluster analysis program.

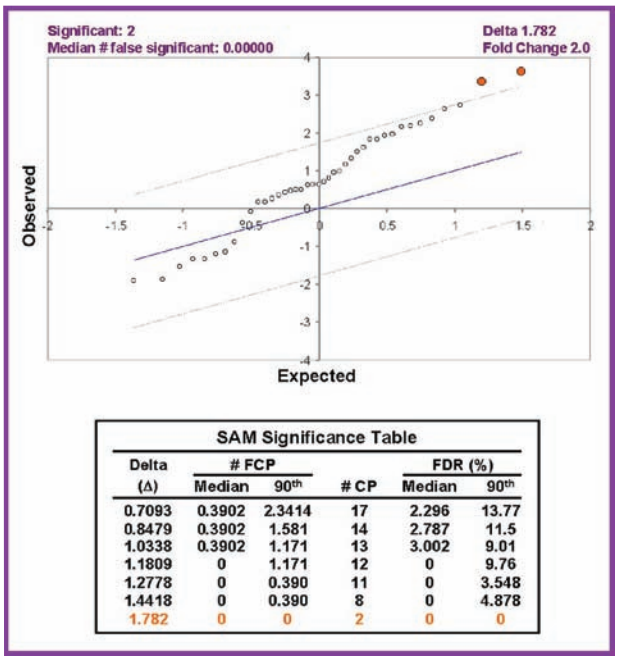


BioMarker Wizard Peak Cluster Summary

Cluster #	Mass	Control Mean	Control SD	Disease Mean	Disease SD	Fold Δ (Dis/ctrl)	*P* value
2	2277.72	10.255	12.574	1.941	2.918	0.19	0.02053
4	2503.32	35.019	17.017	18.517	12.206	0.53	0.02053
7	2836.68	1.201	2.972	7.090	6.556	5.9	0.0433
8	3097.99	17.811	8.953	7.414	7.285	0.42	0.01195
13	3717.51	8.265	4.778	4.084	3.422	0.49	0.0433
18	4600.82	3.568	4.155	19.433	8.414	5.45	0.00264
20	4959.94	3.953	1.228	10.103	4.552	2.56	0.0026
21	4994.23	2.123	2.215	11.065	13.619	5.21	0.00364
23	5108.29	9.13	3.299	15.269	4.147	4.15	0.01195
24	5139.76	10.353	3.769	19.777	6.344	6.34	0.00671
26	5508.89	3.378	3.952	10.309	6.723	3.05	0.0205
30	7353.13	1.774	0.413	9.054	5.773	5.11	0.0036
35	8603.1	5.508	2.74	13.558	6.653	2.46	0.009
37	10264.47	1.29	1.803	14.103	11.662	11.662	0.00264
38	11440.43	0.507	0.281	2.64	2.202	5.01	0.0167

FIGURE 4 - Peak cluster analysis of Au array SELDI-TOF-MS data: Peak cluster analysis (upper panel) was carried out using the Biomarker Wizard tool of the Ciphergen Biosystem software (v3.1.1) to identify common protein peaks across all spectra (total of n = 5 control, and n = 11 disease patients). All spectra were first normalized to total ion current. Peak cluster analysis was then performed for all m/z peaks between 2 and 20kDa with a S/N ≥ 2. The results are represented as a Biomarker Peak Cluster plot (Log normalized peak intensities versus m/z). The table in the lower panel lists the number of significant common peak clusters identified using the BioMarker Wizard program, and the associated mean and standard deviation of the mean (SDM) values for each peak cluster.

Ciphergen Bio systems Inc.) of surgical specimens (disease (DC lesions) and control (unaffected palmar fascia) from the same patient) using a ZipTip$_{C18}$ aided SELDI gold (Au) array approach (i.e. MALDI-TOF-MS, Fig. 2), uncovered several disease associated protein peaks (Fig. 3,4). In total, 15 of 42 identified common peak cluster intensities within the 2 - 20kDa range were identified as being either significantly higher (12 peaks) or lower (3 peaks) within the disease group (n = 13) compared with control (i.e. unaffected) palmar fascia (n = 5). The protein spectral patterns were very reproducible with an average peak cluster mass standard deviations of 1.73 x10^{-4}. Further data analysis was carried out by filtering the common peak cluster spectral data through the bootstrap statistical program called SAM (Significant Analysis of Microarrays).[19] This robust statistical method uses repeated permutations of the data to determine if the expression of any gene/protein are significantly related to a response variable(s), while permitting the user to define both a significant cut-off (Δ value, based on the false positive or discover rate, FDR) and a fold



SAM Significance Table

Delta	# FCP			FDR (%)	
(Δ)	Median	90th	# CP	Median	90th
0.7093	0.3902	2.3414	17	2.296	13.77
0.8479	0.3902	1.581	14	2.787	11.5
1.0338	0.3902	1.171	13	3.002	9.01
1.1809	0	1.171	12	0	9.76
1.2778	0	0.390	11	0	3.548
1.4418	0	0.390	8	0	4.878
1.782	0	0	2	0	0

FIGURE 5 - SAM analysis of Au array SELDI-TOF-MS data: Common peak cluster data were filtered using the SAM bootstrap algorithm (SAM plot, upper panel). SAM uses repeated permutations (n = 5000) of the data sets to determine whether the expression of any protein is significant. Parameters for analysis included a user-defined threshold for significance, or Δ value (Delta = 1.75), and a user-defined fold-change (± 2.0 fold). Using these parameters SAM identified two proteomic features (4600.8 and 10254.5 Da peaks) that are different at a median false significant value < 0.0001, with a corresponding % false discovery rate (FDR, # falsely called protein / # differentially proteins in original data set) of 0%. The table (lower panel) lists the number (median and 90th percentile) of falsely called proteins (# FCP), total number of called proteins (# CP) and the resulting FDR (%) associated with each chosen Δ value.

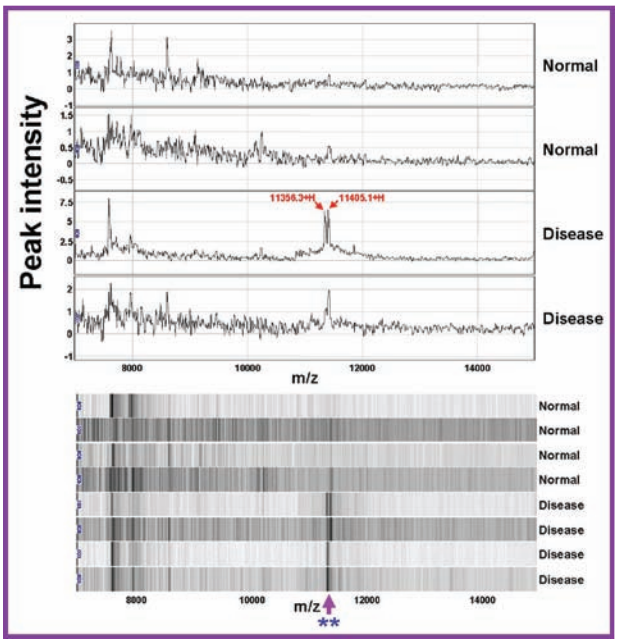


FIGURE 6 - SELDI-TOF-MS analysis of DC using WCX2 ProteinChip® arrays: Protein (5µg) extracted from normal and disease fascia was applied to the active spots of a pre-equilibrated weak cation exchange (WCX2) Ciphergen ProteinChip® array. The spectral data corresponding to peak intensity versus m/z were generated using 65 laser shots per laser intensity setting. Shown above are representative peak intensity spectra (upper panel) and corresponding pseudo-gel views (lower panel) of 4 control and 4 disease patients. *Arrows show two protein peaks that were identified as significantly elevated in the disease tissue using the BioMarker Wizard peak cluster analysis program.

change (ensure level of expression of the called genes/proteins changes a prescribed amount) parameter (Fig. 5). Using stringent screening parameters (0% median and 90th percentile FDR, and a fold change value of ±2 fold) SAM identified 2 significant protein peaks within our peak cluster data set (4600.8 Da, $p4.6^{DC}$; and 10254.5 Da, $p10^{DC}$). These two proteomic features were found to be elevated 5.45 and 11.7 fold, respectively, within the disease group using the Biomarker Wizard peak cluster analysis.

Identification of DC PBM using WCX2 array SELDI-TOF-MS analysis: Peak cluster analysis (Biomarker Wizard program, Ciphergen Biosystems Inc.) of disease and control surgical specimens using the WCX2 array SELDI-TOF-MS approach (Fig. 1) identified several disease associated protein peaks (Fig. 6,7). In total, 2 peaks (11358.2 and 11405.1 Da) out of a total of 19 common peak clusters within the 2 - 20kDa range were identified as being significantly higher within the disease group (n = 10) compared with controls (n = 4). The protein expression patterns were very reproducible with an average peak cluster mass standard deviations of 1.5982 $x10^{-4}$. More stringent bootstrap analysis of the common peak cluster data using SAM identified 1 proteomic feature

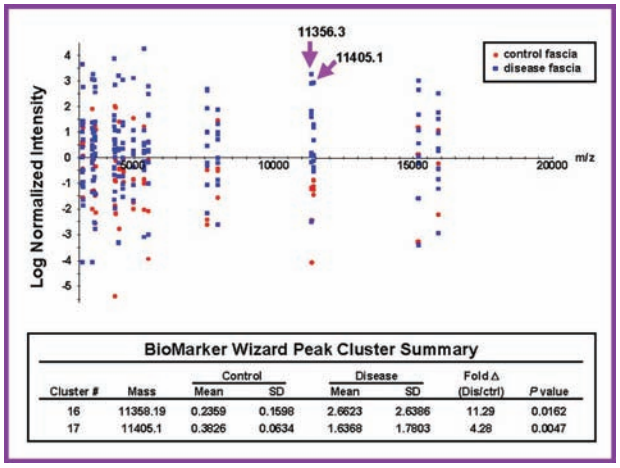


FIGURE 7 - Peak cluster analysis of WCX2 ProteinChip® SELDI-TOF-MS spectral data set: Peak cluster analysis (upper panel) was carried out using the Biomarker Wizard tool of the Ciphergen Biosystem software (v3.1.1) to identify common protein peaks across all spectra (total of n = 5 control, and n = 11 disease patients). All spectra were first normalized to total ion current. Peak cluster analysis was then performed for all m/z peaks between 2 and 20kDa with a S/N ≥ 2. The results are represented as a Biomarker Peak Cluster plot (Log normalized peak intensities versus m/z. The table l (lower panel) lists the number of significant common peak clusters identified using the BioMarker Wizard program, and the associated mean and SDM values for each peak cluster.

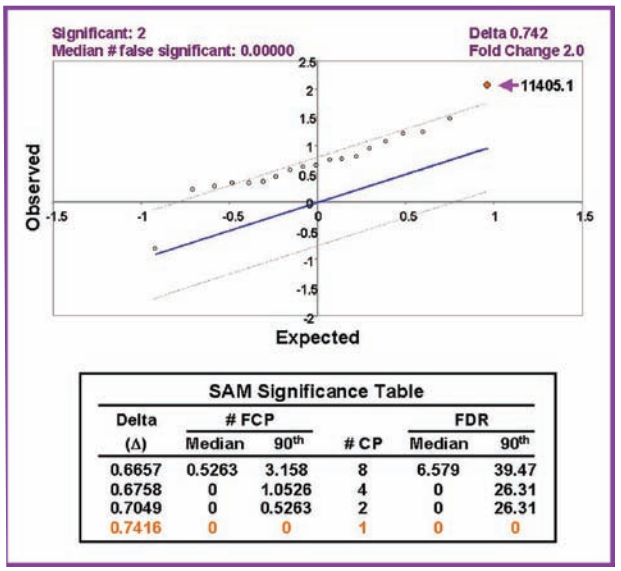


FIGURE 8 - SAM analysis of WCX2 ProteinChip® peak cluster data: Common peak cluster data generated using the Biomarker Wizard program was filtered through the SAM bootstrap statistical analysis program to generate a SAM plot (upper panel). Our chosen parameters were Δ = 0.742, 5000 permutations and a fold change of ± 2 Using these parameters SAM identified one proteomic feature (11405.1 Da peak) that was different at a median false significant value < 0.0001, with a corresponding FDR = 0%. The table (lower panel) lists the number (median and 90th percentile) of FCP, total number of CP and the resulting FDR (median and 90th percentile) for each user-defined Δ value.

(11405.1 Da, p11.4^{DC}) that was elevated (4.28 fold) within the disease group with a median and 90th percentile FDR of 0% (Fig. 8).

DISCUSSION

Dupuytren's contracture (DC) is a debilitating disease of the hand that leads to finger contractures and loss of hand function and is considered one of the most common fibroproliferative connective tissue diseases. It has a high prevalence in Caucasians of Northern European descent, with a reported incidence ranging from 10-40% for males over the age of 60.[20] Unfortunately, at present, DC is incurable and the only (temporary) relief for patients is through surgical resection, an approach virtually unchanged since the middle of the 19th century.[21] Surgical resection, however, has a high recurrence rate , carries risks, is painful and requires prolonged post-operative rehabilitation.[22] DC has a number of remarkable characteristics, which suggest that it is part of continuum of disorders associated with dysregulated cell growth and proliferation. For example, patients with DC have an increased incidence of both benign and malignant neoplasia.[23-25] Also, histologically and biochemically, DC and wound granulation tissue share many similarities, leading to the common belief that DD may be an exaggerated, dysregulated fibrotic wound healing response.[26, 27] Therefore, elucidation of the molecular pathogenesis of DD may also have added clinical significance for the diagnosis and treatment of these other pathological conditions. Unfortunately for the clinician-investigator, it is an intimidating task to find a place to begin unravelling the molecular abnormalities of health and disease.

The advent of high-throughput analytical technology to pinpoint molecular abnormalities gives

the investigator the opportunity to rapidly focus on potential targets for further study. In this study, we have employed a highly sensitive TOF-MS technique to identify changes in protein expression associated with DC.

SELDI-based proteomic profiling of DC specimens resolved an average of 35 common low molecular weight (2 - 20kDa) tissue derived protein/peptide clusters using a $ZipTip_{C18}$ aided Au array based SELDI-TOF-MS approach (Fig. 2,3,4,5), and an average of 29 common low molecular weight (2 - 20kDa) protein/peptide clusters using WCX2 array based SELDI-TOF-MS (Fig. 6,7,8). The spectra generated using these two TOF-MS approaches were highly reproducible with average peak cluster mass standard deviations $<1.74 \times 10^{-4}$ for both methods. While stringent filtering of the protein cluster data using SAM identified only 3 up-regulated proteins (4600.8 Da, 5.45 fold, 10254.5 Da, 11.7 fold; and 11405.1 Da, 4.28 fold) associated with DC, our initial peak cluster analysis generated a more extensive list of both up- (14) and down-regulated (3) peptides and/or proteins (Fig. 4,7). The presence of numerous differentially expressed low molecular weight proteins is not difficult to comprehend if one considers the nature of the sample being examined in this study. DC lesions are very different structures compared to normal fascia tissue. Moreover, DC lesions are quite dynamic structures undergoing extensive remodelling as the disease progresses. Therefore, one would expect several protein and/or peptide by-products of the disease would be present within these active lesions, and perhaps even in the surrounding environment (e.g. blood). While we did not investigate patient serum, recent SELDI-based studies suggest that low molecular weight degradation products (i.e. peptides) can passively enter the blood stream and bind to carrier proteins such as serum albumin.[28] Peptide markers bound to these carrier proteins potentially possess a half-life many orders of magnitude higher than their free-phase counterparts, which are actively cleared by the kidneys, suggesting that serum albumin bound peptides may represent a 'treasure trove' of clinically useful diagnostic information.[29, 30] The rapidly growing list of low molecular weight (<20kDa) serum markers for various human diseases, including HBV-induced liver cirrhosis [15], prostate cancer [31], ovarian cancer [32], hepatocellular carcinoma [16] and head and neck cancers [14], perhaps best reflects the success of this SELDI-based MS approach. In those studies, the use of various algorithms to analyze the serum protein expression patterns has generated highly sensitive (83-100%) and specific (90-95%) proteomic disease signatures that may prove very useful for early disease detection. In our study, the use of the bootstrap algorithm SAM to filter the protein cluster data helped to further decipher the importance of the peak cluster generated by SELDI-TOF-MS. In particular, SAM permitted us to define both a fold-change parameter to ensure the level of expression of the called protein changes a prescribed amount, and a significance cut-off parameter (delta, Δ) based on the false discovery rate. Although further studies will be needed to identify and validate these potential protein markers, it appears that the use of these novel technologies can help us identifying elements of the human proteome that may be targets for the development of alternative methods for the detection and treatment of this disease.

CONCLUSIONS

Our analysis of DC patient material identified several disease-associated low molecular weight proteins. Peak cluster analysis of the SELDI-TOF spectra coupled with SAM bootstrap filtering of the common peak clusters identified three proteins (4600 Da, $p4.6^{DC}$; 10254.5 Da, $p10^{DC}$; and 11404.2 Da, $p11.4^{DC}$) that were expressed at higher levels within diseased palmar fascia tissue (5.45, 11.7, and 4.28 fold, respectively). The present study provides the first low molecular weight (<20 kDa) proteomic profiling of DC. Although the identity of the differentially expressed proteins and peptides is not known, further analysis using multidimensional SELDI-TOF based approaches, as we have employed before [16, 18] will permit more precise identification of these potential protein or peptide markers. Given the vast clinical potential of the low molecular weight region of the proteome and the inability of more conventional two-dimensional electrophoresis to detect them, it would seem that SELDI-TOF-MS provides a promising new avenue of deciphering the biomarker-rich low molecular weight region of the human proteome. Application of novel technology like this may help clinicians and clinician-scientists focus on specific molecular abnormalities in diseases that have no known molecular pathogenesis, thus potentially uncovering therapeutic and/or diagnostic targets.

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ORIGINAL ARTICLE

Endogenous Hydrogen Sulfide And The Cardiovascular System – What's The Smell All About?

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Abstract

Purpose; Hydrogen Sulfide has been found to be produced endogenously in amounts that cause vasodilatation. The purpose of this review is to outline the effects of hydrogen sulfide as a gasotransmitter.
Methods: This article is a summary of work done in Dr. Rui Wang's laboratory and a comprehensive review of H_2S on the *Pub-Med* website.
Principle Findings: One of the specific cellular targets of hydrogen sulfide is the K_{ATP} channel protein which produces vasodilatation. The other gasotransmitters like NO (nitric oxide) interact with hydrogen sulfide in complex ways. This action could be important in hypertension and other vascular diseases.
Conclusion: Gasotransmitter research is relevant to the pathophysiology of a number of cardiovascular disease processes.

The purpose of this article is to review the basic science of H_2S as a gasotransmitter and to relate this to disorders of the cardiovascular system. Gasotransmitters are small gaseous molecules and are freely permeable to cell membranes. Their action does not rely on the known membrane receptors. These gases can have endocrine, paracrine or autocrine effects. Their production and degradation are balanced to maintain physiological concentrations. At these concentrations they have well defined and specific functions.

In 1992, nitric oxide (NO) was named "molecule of the year" by *Science*. Within six years, Furchgott and colleagues had won the Nobel Prize in physiology or medicine for their elucidation of nitric oxide as a gaseous molecule synthesized by the endothelial cells of the blood vessel wall, causing vasodilatation.[1]. Soon after, Carbon Monoxide (CO) was identified as the second biologically active gaseous molecules.[2]. Work carried out in our laboratory and others has identified H_2S as a third gas that causes blood vessels to relax and dilate.[3] .

Historically, H_2S is best known as a toxic gas – rotten egg gas.[4.5] It can also be generated endogenously from L-cysteine in a reaction catalyzed by cystathionine – beta – synthase (CBS) and /or cystathionine – gamma – lyase (CSE).[6] The detectable level at the nose is 400-fold lower than the toxic level. In aqueous solution, about one third of H_2S remains undissociated at pH 7.4. H_2S solubility in lipophilic solvents is about 5-fold greater than in water.[7] In vascular tissue, H_2S is mainly released from vascular smooth muscle cells rather than from endothelial cells.[3]

The acute toxicity of H_2S has been known for at least 300 years. Acute exposure to H_2S causes four dose –dependent responses: hyperpnea, unconsciousness or knockdown, apnea and death.[8] The scientific mechanisms for these effects are unknown, but inhibition of cytochrome oxidase in the central nervous system by sulfide has been suggested.

Almeida and Guiotti developed an ingenious way to test this premise. They compared the effects of NaHS

(a precursor of H_2S) given intravenously with that given directly into the carotid artery.[9] The intravenous dose of NaHS required to produce apnea was one fifth of that given intra-arterially suggesting that the lung or another peripheral site is the main locus of action of H_2S. When lidocaine was applied to the vagus nerve, apnea was prevented. They concluded that the lung is the peripheral site of action of H_2S. Hyperpnea increased in duration with the dose of NaHS. The carotid body, located distal to the arterial catheter sensed the high doses of NaHS but did not trigger apnea. The following conclusions can be drawn:

i. The carotid body is not implicated in NaHS – induced apnea.
ii. The lung but not the brain harbours the primary site of action of H_2S.
iii. Afferent neural signals from the lung via the vagus induces the apnea. They also causes pulmonary edema as reported by Beauchamp.[10] and Reiffenstein.[11]

The reported toxic level of H_2S is less than two-fold greater than its endogenous level in rat brain tissues.[3] Mammalian cells must, therefore, possess a delicate regulatory mechanism to control the endogenous level of H_2S. Catabolism of H_2S is by oxidation in mitochondria or by methylation in the cytosol, followed by scavenging by metalloproteins, disulfide – containing proteins, thio - s - methyl -transferase and heme compounds.[12]. H_2S is mainly excreted by the kidney as free or conjugated sulfate. H_2S can interact with hemoglobin in forming green sulfhemoglobin.[7] Pretreatment of human erythrocytes with CO to saturate the hemoglobin "sink", results in the accumulation of endogenous H_2S.[13]

Methods

Physiological effects of H_2S on the cardiovascular system

H_2S has cardiovascular effects. It is also physiologically relevant. This is supported by the location of the H_2S – generating enzymes as well as the detection of H_2S in biological fluids. Two enzymes are involved in the endogenous production of H_2S. The expression of CBS and/or CSE is tissue specific.[3] The activity and/or expression of CBS is lacking in the heart and peripheral vascular tissues. CSE has a unique tissue distribution pattern and is not detectable in brain and lungs.[14] CSE is widely expressed in peripheral vascular tissues and the heart. This enzyme has been identified, cloned and sequenced. The message (mRNA) and protein for this enzyme are expressed in vascular smooth muscle cells (VSMC).

Adult human liver exhibits CSE activity, but it is absent from fetal, premature, and full-term neonatal liver tissues. This developmental stage-dependent CSE activity seems to be related to the age-dependent posttranscriptional regulation of this enzyme. Whether there exists a similar age dependent expression of CBS in any tissues has not been determined. H_2S exerts a negative feedback effect on the activity of both enzymes[7]. Another source of H_2S is the non-enzymatic reduction of sulfur to H_2S using reducing equivalents obtained from the oxidation of glucose.[7]

Determination of the circulating level and tissue level of H_2S under physiological conditions is essential for assigning it a physiological role. It has been reported that the endogenous concentration of H_2S is 50-160 μM in rat, human, and bovine brains.[15] Mason et al. reported that the normal whole blood level of H_2S in a Wistar rat is 10 μM.[16] The plasma level of H_2S in Sprague – Dawley rats is 46 μM.[15] In humans, 10-100 μM was reported.[17]

Zhao et al first cloned CSE from rat mesenteric artery.[3] The full sequence of this clone was identical to the CSE clone derived from rat liver in the same laboratory. They also showed that the H_2S induced relaxation of rat aortic tissue was mainly due to the action of H_2S on smooth muscle cells. This reasoning was reached as denervation or endothelium removal had little effect on the vasorelaxation caused by H_2S. In fact, the presence of an intact endothelium augmented the effect of H_2S. Theoretically, this might be because the endothelium retains and prolongs the effect of H_2S in the blood vessel wall.[7]

Interaction of H_2S with other gasotransmitters

The actions of CO, NO, and H_2S are not redundant. Gasotransmitters may interact with each other. Endogenous production of H_2S from rat aortic tissue is enhanced by NO-donor treatment SNP (sodium nitroprusside). The NO donor also enhances the expression level of CSE in cultured VSMC.[7]

Principle Findings

H_2S is an endogenous opener of K_{ATP} channels in VSMC

K_{ATP} channels were originally discovered in cardiac muscle.[18] Later, they were identified in several other tissues.[15] K_{ATP} channels are found specifically in cardiac myocytes, in coronary artery smooth muscle cells as well as in nodal tissues and Purkinje fibres.[19,20,21,22] Patch-clamping techniques showed an increase in K_{ATP} channel current in rat aortic smooth muscle cells after exposure to 300 μM H_2S.[3] This effect was

nullified immediately after washing out H_2S from the bath solution. The interaction of H_2S with known K_{ATP} channel modulators was examined to elucidate whether an ATP-sensitive potassium channel (K_{ATP}) was the target of H_2S. Pinacidil (K_{ATP} channel opener) also increased K_{ATP} channel currents similar to the effect of H_2S,[3,23] Pretreatment with glyburide (K_{ATP} channel blocker) antagonized the hypotensive effect of H_2S. Increased K_{ATP} channel current by H_2S leads to membrane hyperpolarization resulting in smooth muscle relaxation.

The effects of H_2S on the heart

The H_2S production rate of rat myocardial tissue is about 19 nmol/g/min. These results demonstrate that the heart is an important source of endogenous H_2S generation.

CSE mRNA level in the myocardium was 24% higher than that in the thoracic aorta but 14% lower than that in the brain tissue.[12]

NaHS, in a concentration of 10^{-6} – 10^{-4} mol/L, caused negative inotropism but had no effect on heart rate (HR) or coronary perfusion flow (CPF). With a higher concentration of 10^{-3} mol/L both HR and CPF were inhibited in addition to severe negative inotropism.[12]

At 10^{-6} mol/L H_2S causes coronary vasodilation. This suggests that there is a narrow window of concentrations of H_2S that cause vasodilation because, at higher concentrations, there is no coronary vasodilation. The inhibitory effect of high dose NaHS on HR, CPF and contractility might be explained by an overall inhibitory toxic effect on all the measured cardiac parameters.[12]

The possible role of H_2S in the pathogenesis of hypertension

There are complex interactions between vasoconstrictive and vasodilatory molecules that have a crucial role in maintaining the balance of the vascular wall.[24] The plasma level of H_2S in SHR (Spontaneous Hypertensive Rats) group was much lower than that of the WKY (Wistar Kyoto rats) group (20.35 ± 9.2 *vs* 48.40 ± 13.4 µM).[25]. If the circulating concentration of H_2S were diminished because of a lower CSE activity, vasoconstrictor forces should overcome vasodilatory forces. The endogenous H_2S/CSE system is one of the key factors in maintaining basal systolic blood pressure. The deficit of H_2S/CSE system may be responsible for the development of spontaneous hypertension accompanying aorta remodeling and atherosclerosis.

Yan et al demonstrated that gene expression of CSE and the activity of CSE in the thoracic aorta were suppressed in SHR.[25] Elevating plasma level of H_2S also attenuated the elevation of blood pressure and lessened the aorta structural remodeling (as measured by aorta medial cross-sectional area and medial stress forces) during the development of hypertension.

An *in vivo* rat study demonstrated that H_2S has a hypotensive effect on blood pressure.[3] H_2S provoked a transient (30 sec.) decrease in mean arterial blood pressure by 12 – 30 mm Hg in anesthetized rats. In addition, central venous pressure was also decreased by H_2S from a normal of 4.2 mm Hg to 1.67 mm Hg.[3,25]

Other pathophysiological conditions:

In a rat model of septic and endotoxic shock the authors measured hemodynamic variations, metabolic data, H_2S and NO content of different arteries. The HR (heart rate), MAP (mean arterial pressure) and contractility decreased markedly while LVEDP (left ventricular end diastolic pressure) increased. Arterial H_2S content was increased in both septic and endotoxic shock. It was suggested that endogenous H_2S was involved in physiological and pathophysiological process during shock.[26] This is akin to nitric oxide: high levels caused by inducible NOS lead to vasodilatation and low blood pressure in sepsis.[26]

Hyperhomocysteinemia is a disease characterized by deficient expression of CBS which leads to premature peripheral and cerebral occlusive arterial disease.[27] The role of a low level of endogenous H_2S in the pathogenesis of this disease has been largely overlooked or neglected, yet it may be an important cause of atherosclerosis and thrombotic complications associated with hyperhomocysteinemia.[27]

The development of vascular diseases after heart transplantation is accompanied by increased total plasma homocysteine concentration.[28] In this case and other vasculopathy circumstances, a potentially lower endogenous level of H_2S may be an important pathogenic factor.

The pathophysiology of the H_2S gasotransmitter in hypertension and vascular vessel wall remodeling has been explored. Other clinical conditions will be related to the H_2S/K_{ATP} channel interaction. Molecular mechanisms of the interaction of H_2S and K_{ATP} channels in other tissues should be further investigated.

Discussion

The literature suggests that H_2S is a biologically active molecule which has a vasodilatory effect. Abnormal

function or metabolism of this gasotransmitter may be related to the pathophysiology of cardiovascular diseases processes. We have compiled evidence that suggests that H_2S is endogenously generated and metabolized in the body and that it acts via K_{ATP} channels to cause blood vessel vasodilatation. This effect is important and reproducible. Future studies should further address the effect of H_2S on the tone of the blood vessel wall.

We conclude that H_2S is not just a toxic gas; but that it is endogenously generated and has vasodilatory effects at physiologically relevant concentrations. H_2S has an effect on K_{ATP} channels (target protein) in VSMC. The current H_2S investigation may exert great impact on many conventional doctrines and thinking about signaling molecules. A few known diseases, such as hyperhomocystinemia and hypertension are potentially related to the abnormal interaction of H_2S with its cellular targets.

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ORIGINAL ARTICLE

The importance of foreign-trained physicians to Canada.

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Abstract

Purpose: To examine the proportion of Canada's physicians who are foreign-trained (non-Canada, non-US), and to determine if there was a relationship between this number and the net change in physicians of each province as affected by inter-provincial migration.

Methods: Data were obtained from the Canadian Medical Association, based on information contained within the Southam Medical Database of the Canadian Institute for Health Information (1987-2003). Information on the net change in the number of physicians lost or gained due to inter-provincial migration was obtained for each province, as well as the percentage of physicians that are foreign-trained (non-Canada, non-US). A correlation between the net change in physician supply and the proportion of foreign-trained physicians was explored.

Results: Foreign-trained physicians comprised from 19% (Prince Edward Island) to 55% (Saskatchewan) of the provincial physician supply. There was a strong linear correlation between the net change in physician supply due to inter-provincial migration and the proportion of foreign-trained physicians (r^2 0.546; P=0.0146).

Discussion: Canada continued to rely heavily on foreign-trained physicians. This was particularly true for provinces which lost the greatest number of physicians to inter-provincial migration. Such 'poaching' of physicians may have important ramifications for the source countries.

Previous work has shown that Canada aggressively recruits foreign-trained physicians, particularly from South Africa[1]. This has led to an intense debate of Canada's social responsibilities: should Canada place its own people first by recruiting much-needed physicians from other countries, or should Canada dissuade highly-trained physicians from leaving their native country?

The objectives were to determine the proportion of Canada's physician workforce that continued to be foreign-trained. Also, we examined the relationship between the number of physicians who have moved from one province to another to practice, and the percentage of physicians in practice who were trained in a country other than Canada or the United States. The original hypotheses was that Canada continues to rely heavily on foreign-trained physicians, and that provinces which suffered the greatest "loss" of physicians due to interprovincial migration relied most on foreign-trained physicians.

Methods

The percentage of practicing physicians in each province that were foreign-trained (non-Canadian, non-American; country of MD-graduation) was obtained from the Canadian Medical Association[2], and was based on information contained within the Southam Medical Database of the Canadian Institute for Health Information[3]. The absolute number of physicians that were lost by each province due to interprovincial migration was also obtained from the Canadian Medical Association (Southam Medical Database), and totaled for the years 1987-2003. This number was then divided by the total population of the cor-

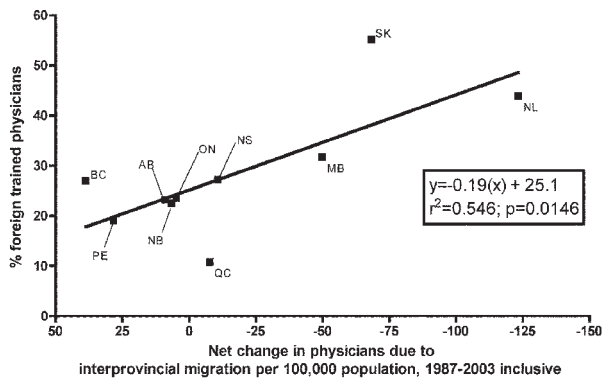

FIGURE 1. Relationship between interprovincial migration of physicians (1987-2003) and percentage foreign-trained (non-Canadian, non-American) practicing physicians.* *Note: Yukon, Northwest Territories, and Nunavut were excluded due to low relative populations; BC=British Columbia; PE=Prince Edward Island; AB=Alberta; NB=New Brunswick; ON=Ontario; NS=Nova Scotia; QC=Quebec; MB=Manitoba; SK=Saskatchewan; NL=Newfoundland; residents and physicians >80 yrs excluded.

responding province in 2003 (Statistics Canada), and multiplied by 100,000 to obtain the net change in physicians from 1987-2003 per 100,000 population.

Results

Foreign-trained physicians continued to represent a large proportion of Canada's physician workforce, ranging from 19% (PEI) to 55% (Saskatchewan) by province. Newfoundland, Saskatchewan, and Manitoba lost the greatest number of physicians due to interprovincial migration, while British Columbia and Prince Edward Island gained the greatest number (Fig 1). Saskatchewan and Newfoundland have the highest proportion of foreign-trained physicians, while Quebec and PEI have the lowest proportion. There is a correlation between the net loss of physicians and the percentage of provincial physician workforce that are foreign-trained (r^2=0.546, *P*=0.0146; Fig 1).

Discussion

The results demonstrate that provinces that are losing the greatest number of physicians per 100,000 population through interprovincial migration also have a greater proportion of foreign-trained physicians. In particular, these are Saskatchewan, Newfoundland, and Manitoba. This is despite the fact that nearly three quarters of the total number of recent immigrants to Canada settle in Toronto, Vancouver, and Montreal as the first, second, and third most popular destination cities[4]. Provinces with the greatest net gain of physicians tend to be the richer provinces - British Columbia, Alberta, and Ontario, while those with the greatest loss tend to be poorer (e.g. Newfoundland). The effect of population size for provinces with smaller populations, particularly Prince Edward Island, is much greater as the denominator is much smaller.

The results point to the continuing importance of foreign-trained physicians to Canadian health care, in particular in Newfoundland and Saskatchewan, and should be considered by those opposed to allowing foreign-trained physicians into Canada. Indeed, this may be one solution to the lack of family physicians within some provinces. By aiding foreign-trained physicians to obtain licenses to practice in regions in need of physicians, Canada may ensure adequate access to health care to its population.

If Canada continues to allow, and even recruits, foreign-trained physicians to Canada, it must be done with care and sensitivity. Attracting highly trained physicians who underwent their medical education in the developing world is controversial, and has repeatedly been discussed in the *Canadian Medical Association Journal*[5]. Should our priority be to ensure our own adequate access to health care, or should we discourage active recruitment of physicians from developing countries, for the good of these countries? Do we have the right to restrict foreigners from practicing in Canada, for the good of *their* country, and against their will?

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SNAPSHOT FROM THE 2004 NATIONAL PHYSICIAN SURVEY

Physician Payment Preferences

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The NPS is a comprehensive questionnaire distributed to medical students, residents and physicians across Canada. Data for payment preference were extracted and categorized by age (<35, 35 – 44, 45 – 54, 55 – 64, and >65 yr), and compared using chi-square analyses.
Data were extracted from 20,272 of 59,399 practising physicians (response rate 34%), including 1,829 <35, 5,294 - 35-44, 6,622 - 45-54, 4,461 - 55-64, and 2,066 aged >65 yr. Older physicians preferred FFS payments compared with younger physicians: 48.5% aged >65 preferred FFS compared with 19.4% < 35yr ($P<0.01$). The majority (58.6%) of younger physicians preferred "blended payments" (i.e., a mixture of FFS and salary based compensation).

[1]Physician census conducted in 2004 by CMA, CFPC, RCPSC: www.nps-snm.ca

PROCEEDINGS REPORT*

The Mediterranean Diet in Secondary Prevention of Coronary Heart Disease

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Abstract

Epidemiological studies as well as randomised dietary trials suggest that Mediterranean diet may be important in relation to the pathogenesis and prevention of coronary heart disease (CHD). A striking protective effect of a Mediterranean diet rich in alpha-linolenic acid (ALA) was reported in the Lyon Diet Heart Study with a 50 to 70 % reduction of the risk of recurrence after four years of follow-up in CHD patients. According to current knowledge, dietary ALA should represent about 0.6 to 1 % of total daily energy or about 2 g per day in patients who follow a traditional Mediterranean diet. Supplementation with very long chain omega-3 fatty acids (c.1g per day) in patients following a Mediterranean type of diet was shown to decrease the risk of cardiac death by 30% and of sudden cardiac death by 45% in the GISSI trial. Thus, in the context of a diet rich in oleic acid, poor in saturated fats and not high in omega-6 fatty acids (a dietary pattern characterizing the traditional Mediterranean diet), even a small dose of very long chain omega-3 fatty acids (one gram under the form of capsules) might be very protective. These data underline the importance of the accompanying diet in any dietary strategy using fatty acid complements.

The concept of the Mediterranean diet originated from several observational studies in the 1950s, the main one being the Seven Countries Study initiated by A. Keys.[1] Taken as a whole, these studies showed that, despite a high but variable fat intake (high in Greece, low in Tunisia, Morocco and Spain, in Catalonia for instance), populations in these countries had low to very low rates of CHD (and other vascular diseases), of most types of cancer and of inflammatory and degenerative diseases. This resulted in long life expectancy. The Mediterranean dietary pattern was considered to be largely responsible for the good health observed in these regions because of the lack of differences in the traditional risk factors, such as smoking, diabetes and high blood pressure, with other less protected populations.[2]

However, despite these strong epidemiological data, causal relationships could be demonstrated only by conducting clinical trials. We now have the results of randomised trials supporting the theory that the Mediterranean diet is a very healthy diet, especially in secondary prevention of CHD.

The Lyon diet heart study

The Lyon Diet Heart Study is a secondary prevention trial designed to test the hypothesis that a Mediterranean ALA-rich diet may improve the prognosis of patients having survived a first acute myocardial infarction.[3-6] The design, methods and results of the trial have been reported.[3-5] A striking protective effect of that Mediterranean diet was reported with a 50 to 70 % reduction of the risk of recurrence after 4 years of follow-up .[3-6] Briefly, as regards lipids, the

experimental Mediterranean diet tested in the trial supplied less than 30% of energy from fats and less than 8% of energy from saturated fats. Regarding essential fatty acids, the intake of linoleic acid (LA), the main omega-6 fatty acid, was restricted to 4% of energy and the intake of ALA, the main omega-3 fatty acid, made up more than 0.6% of energy. In practical terms, the dietary instructions were detailed and customized to each patient[3-6] and can be summarized as: more bread, more cereals, more legumes and beans, more fresh vegetables and fruits, more fish, less meat (beef, lamb, pork) and delicatessen, which were replaced by poultry; no butter and cream, to be replaced by an experimental canola oil-based margarine. Finally, the oils recommended for salad and food preparation were exclusively olive and canola (erucid acid-free rapeseed oil) oils. The scientific rationale for the «dietary fat strategy» has been discussed elsewhere.[3-6] Briefly, it was hypothesized that, because the lowest rates of cardiovascular diseases in the world were observed in populations following either a Mediterranean diet or a diet low in n-6 fatty acids but rich in n-3 fatty acids, the best strategy to reduce the rate of complications in patients with established CHD should be to adopt an n-3 fatty acid-rich Mediterranean diet.

Two other major components of the traditional Mediterranean diet, in addition to a low n-6/n-3 fatty acid ratio, are low saturated fat intake and high oleic acid intake. Thus, to meet the criteria of a Mediterranean diet, patients had to reduce drastically the consumption of foods rich in saturated (essentially animal) fat. Among vegetable oils, only olive oil (despite its lack of ALA) and canola oil (despite its moderate amounts of LA) have a fatty acid composition in line with our strategy. This was well accepted by the French patients who, at the end of the trial, were following a diet whose characteristics were close to the gold standard cardioprotective diet, in terms of lipid nutrients.[3-6]

The exclusive use of olive and canola oils (and of canola-oil based margarine instead of butter to spread on bread) to prepare meals and salad was a major issue in that trial as it resulted in significant differences in the fatty acid composition of both circulating plasma lipids (essentially lipoproteins) and cell membrane phospholipids.[3,7] When comparing the dietary fatty acids in the two groups, control patients consumed about 0.7 g of ALA per day against about 1.8 g in the experimental group, i.e. giving an LA to ALA ratio of about 10 to 1 in controls against about 4 to 1 in the experimental group.

Because the Mediterranean diet tested in that trial was different from the control diet in many other aspects than the LA to ALA ratio (less saturated fat, more antioxidants from various sources, and probably more vitamins of the B group including folic acid, more vegetable proteins, and so on), the next question was to try and specify the exact role of ALA in the cardioprotection observed in the trial. Using multivariate analyses and adjustment for several confounders, we found that the plasma ALA levels measured two months after randomisation were inversely associated with the risk of recurrence, and in particular of fatal recurrence.[6] It could be said, however, that it is not ALA per se that was protective but the very long-chain n-3 fatty acids derived from ALA, eicosapentanoic and docosahexanoic acids (DHA), which were also increased in the plasma of experimental patients.[3,7] These very long-chain n-3 fatty acids prevent ventricular fibrillation (VF) and sudden cardiac death (SCD) in animal experiments[8,9] and in human trials.[10,11] In the Lyon trial, these fatty acids were not associated with a lower risk, which suggests that ALA was the main protective factor. Also, a specific antiarrhythmic effect of ALA itself was reported in the animal studies where it was tested.[9] The potential protective effects of ALA on cardiovascular diseases were discovered when it was reported that populations with a high proportion of ALA in plasma lipids (Greek and Japanese cohorts of the Seven Countries Study) were protected from cardiovascular diseases[12] and that these low-risk populations consume foods rich in ALA.[13,14]

Simopoulos and colleagues showed that many green leafy vegetables, such as purslane, largely consumed around the Mediterranean basin are a major source of ALA for the Cretan population.[13] The Cretan diet is also rich in antioxidants. Because of its three double bonds, ALA is highly sensitive to oxidation and a high intake of ALA must be combined with a high intake of antioxidants to protect it from oxidation. The same authors also reported that eggs from range-fed Greek hens, which make a feast of purslane and other ALA-rich fresh green grass, are richer in ALA and other n-3 fatty acids than eggs bought in US supermarkets which are usually rich in LA.[14] The large difference in the fatty acid composition of the two types of eggs was also due to the fatty acid composition of the industrial feedstuff given to US hens. This suggested that egg yolk might have been a considerable source of ALA and other n-3 fatty acids for people living in the Mediterranean area. This population usually does not consume large amounts of marine products that

are rich in very long chain n-3 fatty acids. Eating purslane, or equivalent ALA-rich leafy vegetables, to obtain large amounts of ALA is not easy because purslane is not available in many areas. In addition, one big portion, 100 g, of purslane provides less than 0.4 g of ALA. Thus, for most adults, purslane should be associated with either walnuts or a salad dressing containing canola oil. One advantage of eating green leafy vegetables is to increase the diversity of the diet, which is probably a major component of any healthy diet and one advantage of eating eggs from hens fed with ALA-rich grains is to increase the intake of DHA, the longest omega-3 fatty acid otherwise only found in fatty fish and fish oil.

Recently, Singh and colleagues reported a randomised trial in the secondary prevention of CHD conducted in South Asian people, a population at a high risk of CHD not explained by conventional risk factors.[15] Most patients were vegetarians and did not eat fish or foods providing them with very long chain n-3 fatty acids. The experimental group ate more fruits, vegetables, legumes, walnuts and almonds than did controls. Also, the experimental group had an increased intake of whole grains and mustard or soybean oil that are rich in ALA. The investigators calculated that the mean intake of ALA was twice as high in the experimental than control group (1.8g vs. 0.8g) and total cardiac endpoints were fewer (39 vs. 76 events) in the experimental group. This confirmed the Lyon Study as the trial was conducted in a very different social and ethnic context. A group of Indian and US investigators recently reported data similar to those of Singh and collaborators. They insisted on the importance of vegetables and ALA-rich oil (mustard oil is the oil preferentially used by Indians) as major contributors to the lower risk of CHD among Indians.[18]

Further work is needed to support the theory that a cardioprotective diet rich in n-3 fatty acids, can be rich in either ALA or very long chain n-3 fatty acids (EPA+DHA) or, alternatively, rich in either EPA or DHA. The underlying question is whether the different major n-3 fatty acids, ALA, EPA and DHA, individually have the same cardioprotective properties. Another unsolved question is what is the effectiveness of the endogenous synthesis of DHA from EPA and ALA; and whether this synthesis occurs in the same way in the different organs in humans. Recent data suggest that in humans, if any synthesis of DHA occurs from EPA or ALA, it is in small amounts.[3,7,16,17] Our last question for future work is whether an increased intake of ALA and DHA, in the context of a Mediterranean diet, could be more protective than a simple increased intake in ALA, as we did in the Lyon trial.[3,7]

Fish Oil and Very Long Chain n-3 Fatty Acids in Clinical Trials

The theory that eating fish, a major component of the traditional diet of several Mediterranean populations (e.g. South Italy, Sicily, Catalonia and Portugal) may protect against cardiac death is derived from a secondary prevention trial, the Diet And Reinfarction Trial (DART), that showed a reduction in total and CV mortality (by about 30%) in patients who consumed at least two servings of fatty fish per week.[10] The authors suggested that the protective effect of fish might result from a preventive effect on ventricular fibrillation (VF), since no benefit was observed in the incidence of nonfatal AMI. The hypothesis was consistent with experimental evidence suggesting that the very long chain n-3 fatty acids, the dominant fatty acids in fish oil and fatty fish, have an important effect on the occurrence of VF in the setting of myocardial ischemia and reperfusion in various animal models, both in vivo and in vitro.[8] Recently, Billman and colleagues demonstrated, in a dog model, a striking reduction of VF after intravenous administration of pure n-3 fatty acids, including the very long chain fatty acids present in fish oil and alpha-linolenic acid, their parent n-3 fatty acid occurring in some vegetable oils.[9]

Support for the hypothesis of an antiarrhythmic effect of n-3 fatty acids in secondary prevention of CHD, as put forward in DART[10], came from epidemiological studies. In the case control Seattle study, the consumption of fish in both the cases and controls was very low, again supporting the theory that in the Western population there is a deficiency in omega-3 fatty acids.[18] Finally, in a large prospective study (> 20 000 participants with a follow-up of 11 yr), Albert et al examined whether fish might have antiarrhythmic properties and prevent SCD.[19] They found that the risk of SCD was 50% lower for men who consumed fish at least once a week than for those who had fish less than once a month. Interestingly, the consumption of fish was not related to non-sudden cardiac death, which suggests that the main protective effect of fish, or very long chain n-3 fatty acids, is related to an effect on arrhythmia.

The GISSI-Prevenzione trial was aimed at addressing the question of the health benefits of foods rich in very long chain n-3 fatty acids (and also in vitamin E) and their pharmacological substitutes.[11] Patients

(n=11,324) surviving a recent AMI (<3 months) were randomly assigned supplements of n-3 fatty acids (1 g daily), vitamin E (300 mg daily), both or none (control) for 3.5 yr The primary efficacy endpoint was the combination of death and non-fatal AMI and stroke. Secondary analyses included overall mortality, CV mortality and SCD. Treatment with n-3 fatty acids significantly lowered the risk of the primary endpoint (the relative risk decreased by 15 %). Secondary analyses provided a clearer profile of the clinical effects of n-3 fatty acids. Overall mortality was reduced by 20% and CV mortality by 30%. However, it was the effect on SCD (45 % lower) that accounted for most of the benefits seen in the primary combined endpoint and both overall and CV mortality. There was no difference across the treatment groups for nonfatal CV events, a result comparable to that of DART.[10] In that trial from Italy, all patients were advised to follow a Mediterranean type of diet after their AMI. In their report, the GISSI investigators confirmed that the patients of both groups did so. More than 80% reported that they consumed olive oil every day.[11] Thus, in GISSI, the prevention of SCD resulting from the consumption of 1 g very long chain n-3 fatty acids was observed in patients following a Mediterranean diet as background diet. Whether they would have been protected in the same way with a non-Mediterranean diet is unknown. Another way of seeing the question is to ask whether or not the GISSI patients were relatively deficient in very long chain n-3 fatty acids. If they were, we can understand that even a small dose of n-3 fatty acids was so effective. Recent, unpublished data from the European IMMIDIET Project suggest that the Italian population could be relatively deficient in n-3 fatty acids compared with British (South of London) and Belgian (Flemish) populations, thus confirming the hypothesis that a low dose of n-3 fatty acids was so effective in GISSI because the tested population was probably rather deficient in n-3 fatty acids. In the context of a diet rich in oleic acid and poor in saturated and n-6 fatty acids, even a small dose of n-3 fatty acids (under the form of capsules) might be very protective. It is uncertain whether the results would have been similar in another context, for instance in populations with high intake in n-6 fatty acids. Finally, in a separate analysis, the investigators of GISSI analyzed in their cohort, pooling the randomized groups, the effect of adhering closely or not to the main Mediterranean diet principles.[20] They found that, compared with patients in the worst Mediterranean diet score, the risk of dying (from any cause) for those in the best score was reduced by 50% after adjustment for age, sex, smoking, concomitant drug therapy and randomized treatment [20], similar to the Lyon trial. This was confirmed by a recent epidemiological study from Greece again which showed that following a Mediterranean diet considerably reduces the risk of premature death.[21] Indeed, Trichopoulou has reported, using a food-frequency questionnaire and a Mediterranean diet scale (higher score indicating a greater adherence to the Mediterranean diet) in more than 22,000 adults in Greece, a reduction in total, cardiac and cancer mortality with a high degree of adherence to the Mediterranean diet.[21] The data are in line with previous observational and trial data. For all these reasons, several national and international expert Committees, notably in Europe and USA [22], have decided to definitely adopt the Mediterranean diet as the reference diet for the prevention of cardiovascular diseases.

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PROCEEDINGS REPORT*

Mechanical airway clearance using the Frequencer electro-acoustical transducer in cystic fibrosis*

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Abstract

Purpose. Clearance of mucus from airways is the cornerstone of therapy for lung disease in patients with cystic fibrosis (CF). This paper describes the operation of the Frequencer, a novel respiratory physiotherapy device comprised of an electro-acoustical transducer. We hypothesized that the Frequencer would be a safe and effective therapy to help clear secretions from the airways of subjects with CF.
Methods. To verify this hypothesis, 22 individuals with CF were recruited to this study comparing sputum production during conventional chest physiotherapy (CCPT) and Frequencer therapy using a crossover design. The sputum weight was the main outcome measure.
Results. Sputum weight was found to be a reproducible measure of the efficacy of chest physiotherapy in individual patients. The Frequencer induced airway clearance in patients with CF that was equivalent to that of CCPT. Furthermore, treatment of a 4% mucin preparation ex vivo with the Frequencer significantly reduced the viscosity of the mucin solution as determined in a capillary rheometer.
Conclusions. These results indicate the Frequencer is safe and as effective as CCPT in inducing airway clearance in patients with CF.

Bronchial mucus exists in two layers, a low viscosity periciliary liquid (PCL), and above this a more viscous mucus layer,[1,2] The elasticity of mucus appears to change with the rate of application of stress to it, and may be important to the rate of beating of bronchial epithelial cilia. Excessively large quantities of secretions are present in the airways of patients with bronchiectasis particularly in those with cystic fibrosis (CF).[3]. Furthermore, in CF the PCL layer is thinner, and the thicker mucus layer tends to impede the movement of the cilia.[4]. In normal humans, the concentration of mucins in the mucus may be as high as 1% by weight.[5] In CF, the mucin concentration of mucus increases due to excessive transepithelial water absorption. This serves to increase viscosity, reduce elasticity, and increase adhesiveness, which hinders its transport.[6]. The absolute viscosity of normal mucus is typically 1 Poiseuille, rising as high as 500 Poiseuilles in CF patients.[7].

FIGURE 1. Illustration of the Frequencer. The power head to the left is controlled by an amplifier unit to the right, which allows one to adjust both the power and the frequency of the output signal. The results of the study reported in the current manuscript were obtained using a prototype of the illustrated apparatus.

Mucus is a viscoelastic gel termed a Maxwellian liquid because its stress-strain curve is not linear.[8]. Upon application of stress, mucus initially stores energy but with continued stress it will begin to flow like liquid. When the flow stops, it returns to its original state. A well-known example of a viscoelastic gel is dripless paint. When the paint is pulled along on a surface by a brush or roller, it spreads well. However, when the stretching stops, it "gels" in place. This behaviour, although desirable in paint, can cause problems in mucus clearing. Viscoelastic gels move only when applied stress is greater than a threshold level. If the threshold value of stress required for the flow of very viscous gels is not reached, then the gel will simply deform without flowing. Since airway secretions in patients with CF are particularly viscous, mechanisms applying continued stress are needed to induce airway clearance.[9-15] Acoustic percussion has been suggested as a potential modality that may help induce airway clearance.[6]

The Frequencer is a digitally controlled electro-acoustical transducer device invented recently by Louis Plante, a CF patient. It is manufactured by Dymedso. It consists of a control unit and a power head (Figure 1). The power head transmits sinusoidal mechanical and acoustical vibrations to the airways at various locations in the chest. The Frequencer's transducer is applied to the area of the thorax to be stimulated. The power head in the Frequencer provides both mechanical and acoustical stimulation at an adjustable forcing frequency that is typically between 25Hz and 40Hz. The user adjusts the frequency of the transducer such that it causes a sympathetic resonance felt by the patient within the thorax. In most CF patients, the Frequencer induces coughing, followed by expectoration of sputum. This treatment is repeated at all conventional chest physiotherapy (CCPT) positions for approximately the same time as CCPT. The mechanical impulse is applied to a rigid circular perimeter surrounding an acoustical compression chamber, while the acoustical wave is applied at lower pressure, but spread over the entire surface contained within the said chamber. The impulses are opposite in phase, and are transmitted differently through the tissues of the chest to both surface and deep locations. The control unit houses a digital frequency generator and a highly efficient amplifier.

This novel device imparts a continuous stress to airway mucus through the generation of acoustical waves. We hypothesized that the Frequencer would be as effective as CCPT in helping to clear mucus from the airways of patients with CF. The results of this study were obtained with a prototype of the Frequencer rather than with the illustrated model.

METHODS

Study population. Twenty-two subjects with a diagnosis of CF were enrolled into this study after providing written informed consent. The study was approved by the ethics review board of the Centre Hospitalier Universitaire de Sherbrooke. Patients with an established diagnosis of cystic fibrosis [17] and an FEV1 ≥ 35% of predicted were included in the study. The characteristics of the study population are presented in Table 1. Patients with evidence of respiratory exacerbation within the two weeks preceding the study, or with a history of more than two episodes of severe hemoptysis, defined as > 10 ml of blood, were excluded. No change in antibiotic or other respiratory treatment regime was instituted during the study period.

Validation of sputum weight. The major outcome parameter of this study was sputum weight, which has been suggested to be an appropriate physiotherapy outcome measure in CF patients.[18] In order to validate sputum weight as a reproducible outcome measure in CF patients, eight CF patients underwent two 20 min CCPT sessions at one week intervals. The amount of sputum produced during each therapy and in the 5 minutes following therapy was determined by weight.

TABLE 1. Characteristics of the study population.

Male/Female	11/11
Age (yr)	27.5 ± 1.4
Height (cm)	162.2 ± 2.0
Weight (kg)	54.9 ± 2.0
BMI (kg/m^2)	20.8 ± 0.4
FEV_1 (% predicted)	57.8 ± 3.9
Chronic inhaled antibiotic therapy (n)	15
Chronic azithromycin therapy (n)	7
Pulmozyme aerosol therapy (n)	2

Study design. All patients underwent CCPT for 20 min with clapping and postural drainage. Sputum was collected during CCPT and during the 5 min following this therapy. All collected sputum was weighed. The Frequencer therapy was applied for a total time of 20 min, with applications of 5 min to the anterior and posterior areas of each hemi-thorax. All Frequencer treatments were done with the patient sitting, and no attempt was made to combine the Frequencer with postural drainage. As with CCPT, sputum was collected during the 20 min of treatment and the 5 min following the end of treatment and weighed. The CCPT and Frequencer therapies were separated by 12 – 24 h in each patient, with 11 receiving the CCPT first, and 11 the Frequencer therapy first.

Effect of the Frequencer on mucin rheology in vitro. To determine whether the mechanical vibrations generated by the Frequencer were sufficient to alter mucin rheology, mucin (Sigma-Aldrich, St. Louis, MO) was prepared at a concentration of 40 mg/ml in phosphate buffered saline, pH 7.4. The mucin solution was then divided into three fractions each placed in 1.5 ml polypropylene tubes. The first fraction did not undergo further treatment. The second was exposed to mechanical agitation applied with a vortex stirrer (Vortex-Genie II, Fisher Scientific, Nepean, Ontario, Canada) set at force 4 for 5 sec. The third fraction was placed on a membrane at the Frequencer's power head and the frequency was set to 40 Hz with 50% maximum power output for 5 sec. All three samples were then tested in a capillary rheometer comprised of a 10 µl borosilicate glass micropipette (catalog no.: 21-164-2C, Fisher Scientific). A mucin sample of 5 µl was loaded into the capillary and subjected to a pressure of 0.5 cm H_2O. Results are expressed as the flow rates of the mucin solutions. Each test was repeated eight times.

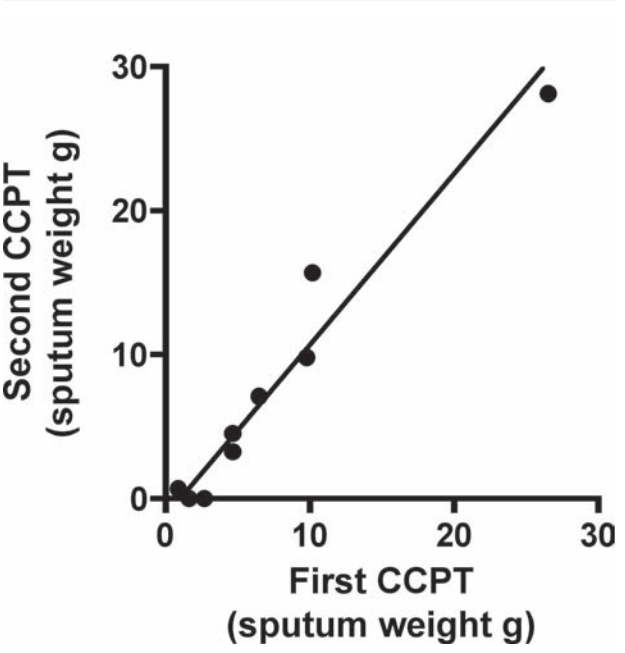

FIGURE 2. Comparison of the reproducibility of sputum weight as a measure of conventional chest physiotherapy efficacy (CCPT). Each patient with cystic fibrosis underwent a first CCPT treatment (abscissa) during which sputum was collected and weighed. One week later the same patients underwent a second CCPT treatment (ordinate) and the results are plotted as a function of the sputum weight from the first CCPT.

Statistical analysis. Data are expressed as the mean ± sem unless otherwise indicated, and the null hypothesis was tested with a paired t-test. Deming linear regression analysis was used to assess the correlation between sputum weights obtained by CCPT at different times in the same CF patients, and between sputum weights obtained with either CCPT or the Frequencer. Calculation of the power of the study to detect a smallest average difference of 35% CCPT sputum weight with a significance level (alpha) of 0.05 (two-tailed) was done using Statmate-2 software from GraphPad. The rheology data were analyzed by one-way ANOVA with a Bonferonni post-hoc test. A value of $P < 0.05$ was considered significant.

RESULTS

The principal outcome measure for this study was the weight of sputum produced during airway clearance

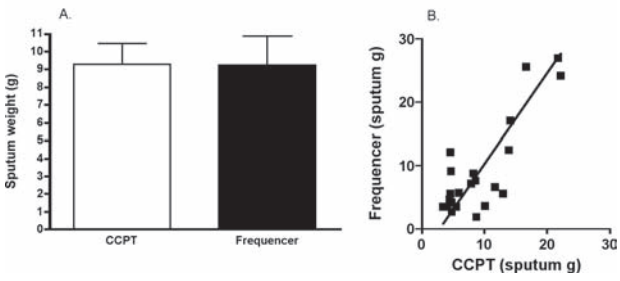

FIGURE 3. Effect of CCPT versus Frequencer therapy on sputum production as determined in 22 patients with cystic fibrosis. All patients underwent both forms of chest physiotherapy for 20 minutes and sputum was collected and weighed during this therapy and during 5 minutes following therapy. Between 18 and 24 hours separated the two therapies. Results are expressed in panel A as the mean ± sem (n = 22, $P = 0.97$) and in panel B with the results of the Frequencer are expressed as a function of those obtained with CCPT. The solid line represents the best fit line created by Deming model II linear regression. The slope is significantly different from 0 ($P < 0.0001$).

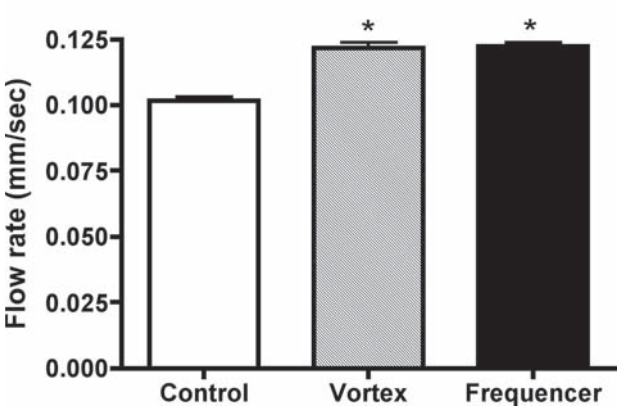

FIGURE 4. In vitro rheological properties of 40 mg/ml mucin solution without (control) and with 5 second treatment using either a vortex stirrer (vortex) or the Frequencer at 40 Hz and 50% maximal power. Results are expressed as the mean ± sem of the flow rate of 5 µl mucin solution through a glass capillary under a constant pressure of 0.5 cm H_2O (n = 8, $*P < 0.001$ vs control).

techniques. The weights of sputum samples produced by each patient during different sessions of CCPT were remarkably similar, (Figure 2) indicating that this measure is reproducible within an interval of one week. Each subject produced similar amounts of sputum during the two CCPT sessions. The median and interquartile range of sputum weight was 4.70 g [2.15 -10.00] for week 1 and 4.60 g [0.35 -12.75] for week 2. The Deming linear regression analysis indicated a slope of 1.182 ± 0.1024 (95% confidence interval 0.94 -1.42) and a correlation coefficient r = 0,98, $P < 0.01$.

As shown in figure 3A, the amount of sputum produced during CCPT was similar to that produced with the Frequencer (CCPT = 9.27 ± 1.20 g *vs* Frequencer = 9.24 ± 1.62 g, $P = 0.97$, n = 22). The mean of the differences in sputum weights between the two forms of therapy was 0.032 g with a 95% confidence interval of -1.85 g to 1.91 g. The Frequencer therapy data plotted as a function of CCPT (Figure 3B) demonstrates a slope of 1.43 ± 0.21 with a 95% confidence interval of 0.99 – 1.87 and a deviation from 0 ($P < 0.0001$). The study had a 95 % power to detect a smallest average difference between pairs of 3.26 g, which corresponds to 35% of the CCPT sputum weight, with a significance level (alpha) of 0.05 (two-tailed).

The application of the Frequencer to a simulated mucus preparation of 40 mg/ml mucin resulted in a significant acceleration of the flow of the mucin solution as measured by a capillary rheometer. The Frequencer-treated mucin preparations reached flow rates similar to those treated with a vortex mixer (Figure 4; control = 1.02 ± 0.01 mm/sec, vortex = 1.22 ± 0.02 mm/sec, Frequencer = 1.22 ± 0.02 mm/sec, $P < 0.001$ each vs. control; $P > 0.05$ vortex vs Frequencer, n = 8).

DISCUSSION

We present evidence that the mechanical and/or acoustical vibrations generated by an amplified low-frequency audio signal at the chest wall can be used to induce sputum production in patients with CF. The Frequencer is a transducer in which an electromechanical process moves a cone to produce sound waves at a power and frequency determined by the user. The study population as a whole did not show any differences in efficacy between CCPT and the Frequencer. However differences in efficacy between these forms of chest physiotherapy were observed in some individuals as is illustrated in figure 3B. Interestingly, it may be possible to predict the efficacy of the different forms of physiotherapy in an individual patient. We found

that the weight of sputum produced by the same CF patient was remarkably similar within a 1 week interval, suggesting that the measurement of sputum weight to compare the efficacy of physiotherapy techniques may be a reasonable approach to choose the best form of physiotherapy for an individual patient.

Therapy with the Frequencer was well tolerated, and in contrast to CCPT, all the Frequencer treatments were done in the sitting position, thus simplifying the therapy. The Frequencer has the potential to provide therapeutic autonomy since the energy is delivered to the chest wall by a device and not by an individual. Since the power and frequency are set prior to therapy, caregivers including parents and spouses do not need to worry about whether the intensity of their therapy is sufficient or excessive. Furthermore, the Frequencer can deliver treatments to specific areas of the chest identified as requiring more intense airway clearance efforts by auscultation and/or by thoracic imaging.

No adverse events were observed with the use of the Frequencer. Several aspects of the Frequencer indicate that it is a safe device. First, the sound pressure level was measured at 56 – 78 dB in free air at a frequency range of 25 - 70 Hz at maximal power, which is well below the threshold for hearing damage in the bass range.[19] Second, stray magnetic fields are minimal, due to the neodymium motor structure. Third, the unit is isolated from electrical shock, and the transducer operates at 20VAC or less. Finally, the constant force applied to the body is about 16 Newtons (N) in order to produce an acoustic seal, yet the Frequencer can provide peak pressures of about twice that of the high frequency chest wall oscillation (HFCWO) device. CCPT (clapping), the current "gold standard" of chest physiotherapy has been measured to deliver 58.10 +/- 15.32 N, at a rate of 6.6 Hz +/-1Hz.[20] The dynamic force applied by the Frequencer's mechanical and acoustical vibrations is between 0.4N and approximately 3N, depending on the degree of coupling between the apparatus and the body. Thus, if clapping is safe, the Frequencer must be much safer. CCPT is a "brute force" method, relying on impact to dislodge mucus, while the Frequencer acts through gentler, metered and targeted mechanical and acoustical vibrations to achieve results.

The mechanisms by which the Frequencer helps to mobilize respiratory secretions are not fully known. However, our in vitro data indicating a more rapid flow rate of a mucin solution after treatment with the Frequencer suggests that the changes in airway secretion viscoelasticity may be one of the mechanisms. These observations are consistent with a study of the Flutter, another physiotherapy device that imparts oscillations to the airway secretions. In a 9-week study, oscillations of CF airways using the Flutter device at frequencies of 22 Hz and less resulted in significantly lower viscoelasticity of sputum in comparison with autogenic drainage.[21] In addition to the reduction of mucus viscosity through repetitive vibrations as described above, other potential mechanisms of action of the Frequencer include shearing at the mucus/airway interface induced by resonant energy targeted to specific locations in the lungs, a combination of mechanical and acoustical coupling from the chest wall that transmits vibrations to small and large airways, peristaltic action due to longitudinal waves, and acoustic streaming and related phenomena. More work is needed to define the mechanisms by which the Frequencer induces airway clearance.

Several options for chest physiotherapy are available to CF patients and their caregivers. A recent analysis of chest physiotherapy publications in the Cochrane database of systematic reviews indicated that while there is no evidence of differences in pulmonary function outcomes between the available techniques and CCPT, participants preferred self-administered airway clearance techniques.[22] Among these techniques were the following: autogenic drainage requiring no device, the PEP-mask device based on positive expiratory airway pressure that favors airway opening and mucus displacement, as well as the Flutter and Acapella which are hand-held devices that use positive pressure and vibration to dislodge airway mucus. Each of these techniques provides autonomy and is relatively inexpensive, but each also requires patient understanding and collaboration, thus limiting usage in young children. Mechanical percussive devices provide more autonomy than CCPT, but can also be associated with discomfort during treatment. More recently, chest physiotherapy applied with a vest that rapidly inflates and deflates to provide HFCWO has gained popularity. Although HFCWO devices provide patient autonomy, they are not recommended for use in infants. In contrast, because the Frequencer does not compress the entire chest wall it may provide an interesting alternative for infants needing airway clearance therapy.

The simplest explanation for the lack of a difference in the efficacy of CCPT and the Frequencer in the current study is that the study may have been under-powered. The study was calculated to have a

95 % power to detect with a significance level of 0.05 a smallest average difference between CCPT and Frequencer of 3.26 g, which corresponds to 35% of the CCPT sputum weight. More patients would need to be studied to detect a smaller difference and it remains possible that the Frequencer may be superior or inferior to CCPT. However, our results are consistent with the concept that both therapies provide equivalent efficacy. The ease of use of the Frequencer and its potential to be used without the aid of a caregiver, provide significant advantages over CCPT.

In addition to being under-powered to detect small differences in sputum production, our study has other limitations. First, the study did not compare the efficacy of the Frequencer with that of other physiotherapy devices and techniques. Second, we cannot predict from this study whether use of the Frequencer will be associated with a significant improvement in clinically meaningful parameters such as quality of life, the number of respiratory exacerbations, and pulmonary function tests. Third, the study was performed using a prototype of the current Frequencer. Since the current model illustrated in figure 1 has an improved power head with a cooling system and a more potent amplifier, better results may be obtained with the current model.

In summary, we have reported a novel physiotherapy device based on the generation of acoustic and mechanical energy that can be delivered to all areas of the chest of patients with disease causing excessive airway mucus production. The device is comfortable, easy to use and safe. Most importantly, it allows the patient autonomy in the delivery of this essential form of therapy. The results presented in this study suggest that the Frequencer is at least as effective as CCPT in facilitating the expectoration of sputum. Further studies will be needed to determine and compare the clinical efficacy of this new form of respiratory physiotherapy delivered alone or in combination with other therapeutic modalities for airway mucus clearance.

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PROCEEDINGS REPORT*

Resistance exercise in chronic heart failure - Landmark studies and implications for practice

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Summary

In patients with chronic congestive heart failure (CHF), there is a need for complementary strength training to maintain and/or increase muscle mass and strength. The challenge is how to stress peripheral muscles intensively without creating cardiovascular overload. Since the late 1990s, an increasing number of research and clinical experiments have been conducted on resistance exercise in CHF. As a result, data are now available for both acute responses during resistance exercise as well as muscular and cardiovascular adaptation to resistance training programs, based on different training methods. Study results demonstrated that dynamic resistance exercise is well tolerated in chronic stable CHF when:

1) initial contraction intensity is low,
2) small muscle groups are involved,
3) work phases are kept short,
4) a small number of repetitions per set is performed, and
5) work/rest ratio is ≥ 1:2.

With tolerance, contraction intensity can be increased. With resistance training programs lasting 12 weeks, maximal strength could be improved by 15 to 50%. Improvements in maximum exercise time and peak VO_2 were between 10 and 18%, in relation to baseline values. In terms of these results, no differences were reported between combined resistance/aerobic training and resistance training alone. Thus, resistance exercise can be assumed as safe as aerobic exercise in clinically stable CHF.

Resistance exercise in chronic heart failure – changing attitudes

Chronic heart failure (CHF) is accompanied by changes in structure and oxidative capacity of skeletal muscles as well as by a reduction of muscle mass and strength. The decrease in total cross sectional area of skeletal muscles and muscle strength is an independent predictor of exercise intolerance and prognosis in CHF. Therefore, resistance exercises and strength training should be included in training programs designed for CHF patients. Since CHF is a progressive disease (5-yr mortality about 50%), substantial increase in muscle mass – normally achieved by performing sports competition or working out in the gym – cannot be expected in CHF patients. Instead, the aim is to delay wasting of muscle tissue. A primary goal is to improve dynamic strength and muscle endurance because of the methods indicated for resistance exercise in CHF (see below).

Until the late 1990s, resistance exercise training was generally considered inappropriate in patients with CHF. This belief was largely based on the extraordinary magnitude of blood pressure response during weightlifting in healthy athletic individuals and on pathologic central hemodynamic measurements obtained during sustained isometric exercise in CAD patients with moderate to severe left ventricular dysfunction: Three to five minutes of handgrip exercise

at only 30% maximal voluntary contraction (MVC) resulted in a marked increase of peripheral vascular resistance, and decrease in left ventricular ejection fraction and stroke work index [1] indicating acute deterioration of left ventricular function during exercising. Over the last few years, attitudes regarding strength training in patients with CHF have changed as a result of hemodynamic measurements obtained during dynamic resistance exercise.

Landmark studies on acute responses to resistance exercise

Cheetham et al. studied patients with severe CHF (NYHA II, III and III-VI) who performed biceps curls and two-footed leg press exercise at 40% MVC for 100 sec with 25 repetitions each.[2] The patients also underwent submaximum cycle ergometry. They found that:

1) Upper and lower body resistance exercise resulted in lower heart rates than submaximum cycle ergometry,

2) The mean arterial blood pressure was similar for resistance exercises and cycling, and

3) During both cycling and resistance exercises, with concomitant decrease in stroke volume, the heart rate started to increase and to enhance cardiac output.

Mc Kelvie et al. studied CHF patients who performed 2 sets of 10 repetitions each at leg press at contraction intensity of 70% of 1RM (1- Repetition Maximum).[3] Cardio-pulmonary measurements obtained during resistance exercise were compared with measurements obtained during graded cycled ergometry. Strength exercises resulted in lower heart rate responses and rate-pressure products than cycling at an intensity of 70% peak VO_2. Left ventricular ejection fraction and left diastolic and systolic volumes were similar during resistance exercise and cycle ergometry.

The first study that focused on invasive central hemodynamics during dynamic resistance exercise in CHF, was conducted by Meyer et al.[4] Resistance exercise was performed at leg press (two-footed; 4 sets of work and rest phases of 60s/120s each; 12 repetitions per working phase; contraction intensity 60% and/or 80% MVC). Patients, NYHA class II and III, exhibited mean left ventricular ejection fraction of 26±3%. They found:

1. Systemic vascular resistance decreased during the course of exercise at 80% MVC load and the amount of decrease was relatively greater than that seen for exercise at 60% MVC load.

2. To demonstrate left ventricular tolerance of resistance exercise left ventricular stroke work index was assessed because it integrates preload, (diastolic pulmonary artery pressur), afterload (mean arterial pressure), and contractility (stroke volume).

3. During resistance exercise at 80% MVC load, stroke work index increased by 23%. Again, the amount of increase was relatively greater than that measured during exercise at 60% load.

These results indicate enhanced left ventricular function during dynamic resistance exercise.

In the study of Karlsdottir et al. echocardiograph measurements were performed in CHF patients (NYHA class II/III; mean ejection fraction 35±5%) while they were exercising at two-footed leg press, shoulder press and biceps curl (sets of 10 repetitions each; contraction intensity 60-70% of 1RM).[5] Also, echocardiographic measurements were performed during a 12-min steady state cycle ergometry at intensity of 90% of ventilatory threshold. For comparison, clinical stable coronary patients with mild left ventricular dysfunction (56±8%) and healthy subjects underwent the same procedure. They found:

1. In CHF patients asked to do cycling exercises, left ventricular ejection fraction increased from 35% to 42% but remained unchanged when they did leg press, shoulder press and biceps curl exercises (between 38% and 35%).

2. Ejection fraction responses indicated no deterioration of left ventricular function during the course of resistance exercise. Besides the clear differences in the absolute value of ejection fraction (with lower values in CHF compared to coronary patients and healthy subjects), there were similar responses within the three groups to different types of exercise and between groups to the same exercise.

In contrast to resistance exercise using sustained muscle contraction, the results indicate that the rhythmic sequence of submaximal muscle contractions taking place during dynamic resistance exercises actually help to reduce systemic vascular resistance; seem to help maintain venous return and peripheral muscle blood flow; and keep left ventricular afterload within tolerable limits. This may be why dynamic resistance exercise is well tolerated by patients with clinically stable CHF.

Intervention studies and resistance training

Ten studies on resistance exercise training have been reported[6-15] (Table 1) involving 242 patients with CHF (mean age 60 yr; mean left ventricular ejection

TABLE 1 Training studies on resistance exercise in patients with stable chronic heart failure

Reference	*Left ventricular Ejection fraktion (%)*	*NYHA Class*	*Duration of training period (Weeks)*	*Contraction intensity*
Resistance exercises plus aerobic training				
Conraads et al. 2002	27	I-II and III-IV	16	50% 1RM [a])
Delagardelle et al. 2002	29	2.7 ± 0.5	24	60-80%1RM
Barnard et al. 2000	25	-	8	60-80%1RM
Maiorana et al. 2000	26	I-III	8	55-65% 1RM
Oka et al. 2000	25	II-III	12	75% 1RM
Hare et al. 1999	26	II-III	8	30-60s/set; low intensity
Magnusson et al. 1996	28 m /11 f	-	8 / 8	80% 1 RM
Resistance exercises				
Selig et al. 2004	27	2.4 ± 0.5	12	30s/set; moderate intensity
Pu et al. 2001	36	2.2 ± 0.1	10	80% 1RM
Tyni-Lenné et al. 2001	30	II-III	8	2 x 25 repetitions; RPE[b]) : 13 on Borg Scale
Average	**26**	**II-III**	**12**	**50-80% 1RM**

a) 1RM = 1 Repetition Maximum b) RPE = Rate of perceived exertion

TABLE 2 Resistance exercise: Criteria to give the right work load, and recommendations reported in the literature

Cardiovascular stress depends on	*Recommendations from literature*
Mode of muscle contraction (sustained - dynamic)	Dynamic
Contraction intensity	50-60% 1RM (MVC) (initially 40-50% 1RM [MVC]) a)
Duration of working phases	≥ 60s
Work: Rest ratio (duration)	≥ 1:2
Number of exercise stations and/or sets	4-6; 1-2
Number of repetitions per set	6-10
Velocity of muscle contraction	6 sec
Involved muscle mass	Segmental → unilateral → two-footed → ....

a. 1RM = 1 Repetition Maximum; MVC= Maximum Voluntary Contraction

fraction 26% [range 11 to 36%]; NYHA class predominantly II and III. In one study of 12 pts, who were NYHA class III-IV] different resistance training methods were used. In eight studies, a combined resistance/aerobic training program was chosen and in three studies only resistance training was chosen. Contraction intensities were mostly set between 50% and 80% of 1 RM. During training periods of 12 weeks (range 8 to 24 wk) patients underwent three training sessions per week in all studies. The results of intervention can be summarized as follows:

1. Maximal strength could be improved by 15% to 50% on average.

2. Improvements of maximum exercise time and peak VO_2 were between 10% and 18%, in relation to baseline values.

3. No differences were reported between combined resistance/aerobic training and resistance training alone.

4. In eight studies, no adverse cardiovascular events, no increased symptoms of heart failure and no need for increased drugs occurred during training and/or were reported within days following the last training session.

5. In two studies one of each of the following adverse events were reported: atrial fibrillation [14] and a sudden death at home three days after the last training.[15] This rate of adverse events is similar to those reported from training programs in CHF that did not include resistance exercises. Based on what we know so far, resistance exercise is as safe as aerobic exercise in stable CHF patients.

Recommendations for strength training

The literature provides many criteria of how to provide the right workload. There is no gold standard and it should be recognised that application of the criteria and their combination (Table 2) in patients with different heart failure pathology, exercise tolerance and training status may result in quite different degrees of cardiovascular strain.

To apply effective stress on peripheral muscles without creating cardiovascular overload, the following rule of thumb may be used: start low – increase slowly. Study results demonstrate that we can expect patients with stable CHF to perform resistance exercises if initial contraction intensity is kept low (e.g. 40% 1RM [MVC]; small muscle groups are involved (segmental muscle training); work phases are kept short; a small number of repetitions per set is performed; and work/rest ratio is ≥ 1:2 (Table 2).

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PROCEEDINGS REPORT*

Synergetic interactions between rehabilitation and pharmacotherapy in COPD

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Abstract

Background. Once viewed as an irreversible condition, chronic obstructive pulmonary disease (COPD) is now considered as a preventable and treatable disease. The past ten years of research have clearly indicate that dyspnea, exercise tolerance and quality of life can be improved considerably with appropriate therapeutic interventions that include pharmacological and non-pharmacological components. It is also becoming evident that it is the concomitant use of appropriate pharmacotherapy and non-pharmacological approaches, such as exercise training and pulmonary rehabilitation, that offers the best hope for an optimal status.
Purpose.The objective of this short paper is to review the rationale of combining pharmacological and non-pharmacological therapeutic approaches to optimize functional status and quality of life in patients with COPD.
Principal Findings: Optimal bronchodilation is the mainstay of treatment. Leg fatigue will prevent patients with COPD from obtaining full advantage of bronchodilation. Quadriceps fatigue during cycling exercise is linked to events taking place within the muscle providing a muscular and metabolic basis to explain the observation that some patients with COPD develop contractile fatigue after exercise. Muscle fatigue can be improved with exercise training. Pharmacotherapy and exercise training offer the best hope for an optimal status in COPD
Conclusion. Our goals are to stimulate interest in COPD, provide a strong case against the nihilistic approach to this disease to ultimately raise the standard of care to the benefit of the numerous patients afflicted by this condition.

Chronic obstructive pulmonary disease (COPD) is characterized by progressive, partially reversible airway obstruction, systemic manifestations, and increasing frequency of exacerbation.[1] This chronic respiratory disease has a profound impact on the life of those who suffer from it. In fact, patients with COPD are often caught in a downward spiral that goes from expiratory flow limitation, hyperinflation, dyspnea, exercise intolerance, and peripheral muscle impairment to poor quality of life and invalidity (Figure 1). Although this disease cannot be cured with current available therapy, it is certainly possible to slow down the progression of a patient within this downward spiral. Despite significant therapeutic advances both in pharmacological and non-pharmacological approaches that make it possible to alleviate shortness of breath and exercise intolerance and to improve quality of life, this disease remains largely undertreated[2] and only a minority of patients have access to optimal therapy as proposed by recently published management guidelines.[1]

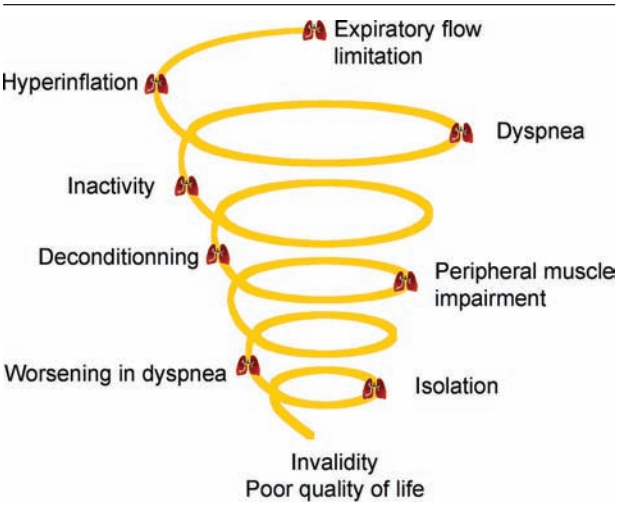

FIGURE 1. COPD downward spiral. Adapted from la Clinique du Souffle la Solane, Osséja, France.

1. Bronchodilation and exercise tolerance

Optimal bronchodilation is the mainstay of the treatment of COPD.[1] Although short acting bronchodilator may be sufficient early on into the disease process, long-acting bronchodilators are best suited to treat chronic symptoms in more advanced disease. Long-acting β2-agonists and once-daily anticholinergic improve dyspnea, quality of life and reduce exacerbations.[3-5] Well designed clinical trials also confirm the efficacy of long acting bronchodilator to reduce operational lung volumes during exercise, translating into less dyspnea and better exercise tolerance as assessed by constant workrate cycling exercise.[6-8]

Despite this convincing evidence, one has to recognize that the impact of optimal bronchodilation alone on quality of life remains modest. A review of recently published clinical trials evaluating the efficacy of the newly developed inhaled therapy for COPD reveals that the impact of these drugs on quality of life barely reaches the clinical significance threshold[9-11] generally defined as an improvement that is noticed by the patient. The discrepancy between the clear improvement in laboratory-based indices of exercise capacity and the relatively modest gain in health-related quality of life is intriguing. It would suggest that the improvement seen in the laboratory does not necessarily translate into better physical function during daily living. One possible interpretation of this phenomenon is that patients may not take full advantage of the improvement gained with bronchodilators unless they are taught to do such as during pulmonary rehabilitation. The interactions between pharmaco-therapy and pulmonary rehabilitation will be discussed later.

Another observation of interest is that the improvement in lung function with bronchodilator correlates only poorly with the changes in exercise capacity.[12] The potential role of leg fatigue in preventing optimal bronchodilation from translating into better functional status is discussed next.

2. Leg fatigue during exercise

Although the exact proportion varies between studies, leg fatigue stands as the predominant limiting symptom at peak cycling exercise in approximately one third of patients with COPD.[13,14] It should be appreciated that peripheral muscle fatigue occurs after whole body cycling exercise in patients with COPD.[15,16] In these studies, the force generated by the quadriceps was evaluated before and after cycling exercise in an effort independent manner using magnetic stimulation. On average, these studies reported a 20% decrease in quadriceps twitch force after cycling exercise in patients who would have traditionally been expected to be primarily limited by early dyspnea and ventilatory parameters. It is worth noting that the occurrence of contractile fatigue after exercise is not in itself abnormal. The problem in COPD is that it occurs at a much lower exercise intensity than in healthy individuals.[17] Patients with COPD are, therefore, disadvantaged while performing daily activities because they are vulnerable to fatigue, even during mildly intense exercise.

We were interested in evaluating whether contractile fatigue of the quadriceps could influence exercise tolerance in COPD.[16] To this extent, 18 patients with COPD were asked to perform, on two separate days, a constant work-rate exercise test until exhaustion. The two constant work-rate exercise tests were preceded by the nebulization of either placebo or ipratropium bromide, in a randomized and double-blind fashion. Using magnetic stimulation of the femoral nerve, the strength of the quadriceps was measured at baseline and after exercise. Half of the 18 patients studied developed contractile fatigue of the quadriceps as defined by a post-exercise reduction in muscle strength greater than 15%. In patients who did not develop fatigue, a 13% improvement in FEV_1 after bronchodilation translated into a better exercise endurance. Conversely, an improvement in FEV_1 of similar mag-

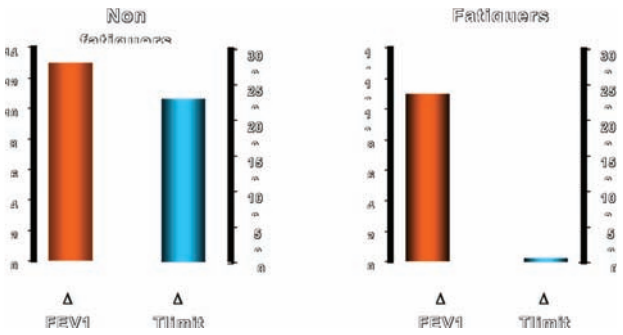

FIGURE 2. Changes in endurance time to constant workrate cycling exercise (Δ Tlimit) in response to bronchodilation according to the presence (fatiguers) or not (non-fatiguers) of contractile leg fatigue. From Saey et al. Am J Respir Crit Care Med 2003;168:425-30.

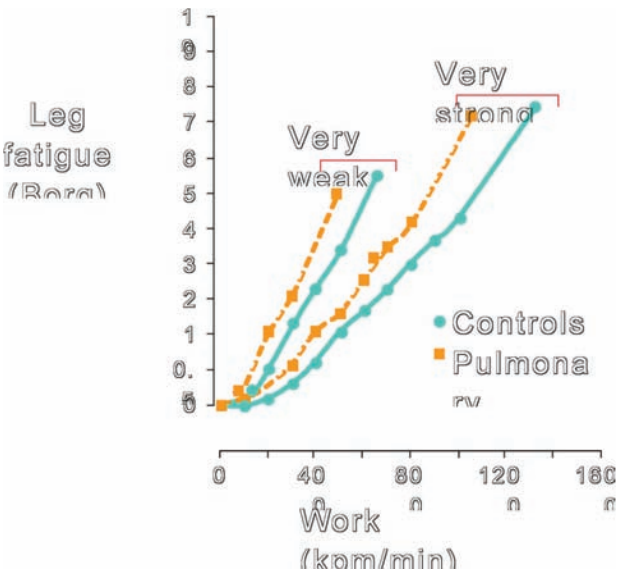

FIGURE 3. Relationships between muscle strength, perception of leg fatigue and exercise tolerance in healthy individuals and in patients with respiratory diseases. From Hamilton et al. Am J Respir Crit Care Med 1995;152:2021-31.

nitude was not associated with any gain in endurance capacity in patients who developed contractile fatigue of the quadriceps (Figure 2). The implication of this study is that the presence of leg fatigue modulates the exercise-response to bronchodilation.

This concept is further supported by a pooled analysis of the two large, multicentre, randomized clinical trials evaluating the efficacy of tiotropium during cycling exercise in patients with COPD.[6,8,18] In this study, the magnitude of the improvement in the endurance time to constant work-rate cycling exercise was smaller in patients whose main exercise limiting symptom was leg fatigue compared to those reporting dyspnea as their primary limitation to exercise.[18] An important clinical message arises from those studies. The occurrence of leg fatigue will prevent patients with COPD from obtaining full advantage of bronchodilation. In these patients, treatment of the peripheral muscles in combination with pharmacological interventions should be incorporated into the management plan.

3. Susceptibility to muscle fatigue

Before addressing how to treat the peripheral muscle, a brief overview of the mechanisms underling fatigue susceptibility in COPD is warranted. Muscle weakness, a common finding in COPD[19-21], is an obvious contributor to muscle fatigue. In a weak muscle, the imbalance between the force necessary to maintain a given contraction and the maximum force will increase the perception of leg fatigue. The relationships between muscle strength, perception of leg fatigue, and exercise capacity were previously investigated in a large group of normal subjects and patients with pulmonary disease, most of whom had COPD.[22] As predicted by the inverse relationship between the perception of muscle effort and muscle strength, the perception of leg fatigue for a given power output, was greater in weak compared to strong individuals. A stronger muscle was also associated with a better capacity to exercise both in normal subjects and in patients with COPD (Figure 3). In fact, in this study, the strength of the quadriceps was found to be a significant determinant of exercise capacity independently from the impairment in lung function.

In addition to muscle weakness, important morphometric changes also predisposing patients to fatigue have been reported.[23,24] A reduction in the proportion of the highly oxidative type I fibre in favour of the more fatigue-susceptible type IIx fibre has been repeatedly reported in COPD. Consistent with structural changes, the activity of important mitochondrial

enzymes is diminished in the quadriceps in patients with COPD compared to healthy controls.[25,26] Muscle capillarization, another determinant of muscle aerobic capacity is also affected in COPD.[23] Overall, these findings indicate that the lower limb muscles of patients with COPD are poorly equipped to face the demand of endurance tasks. The impact of poor peripheral muscle oxidative capacity was further substantiated in a study reporting statistically significant relationships between the endurance of the quadriceps and its enzymatic and fibre-type profile in patients with COPD.[27]

We further evaluated the relationships between muscle profile and fatigue susceptibility in a follow-up study in which biopsies of the quadriceps were obtained from 32 patients with COPD who were *a priori* (based on the post-exercise decrease in quadriceps twitch force) categorized as fatiguers (post-exercise fall in quadriceps twitch force >15%, n = 22) or non-fatiguers (post-exercise fall in quadriceps twitch force ≤ 15%, n = 10)[28]. Differences were found between fatiguers and non-fatiguers in muscle capillarization (lower in fatiguers), muscle LDH activity (higher in fatiguers) as well as in blood lactate response (faster rise in fatiguers) during exercise. Significant correlations were also found between the degree of muscle fatigue on one hand, and muscle capillarization, LDH activity and blood lactate at end-exercise on the other hand. Importantly, there were no differences in fiber-typing or in muscle oxidative enzyme activities between fatiguers and non-fatiguers. Taken together, these data substantiate the hypothesis that quadriceps fatigue during cycling exercise is linked to events taking place within the muscle itself and provide a muscular and metabolic basis for the clinical observation that some patients with COPD develop contractile fatigue after exercise while others do not.

Apart from the reduction in muscle oxidative capacity, other intrinsic muscle factors may also contribute to the development of peripheral muscle fatigue in COPD. Evidence of exercise-induced oxidative stress within the peripheral muscles has been recently reported in patients with COPD.[29,30] One potential implication of this observation is that oxidative stress could predispose muscles to fatigue, as suggested by the inverse relationship between the increase in quadriceps TBARS, a marker of lipid peroxidation during localized muscle exercise, and muscle endurance.[29] These findings support the role of oxidative stress in the development of muscle fatigue and altered endurance capacity in patients with COPD. A role for muscle oxidative stress in explaining muscle fatigue is further supported by a study showing that n-acetyl cysteine supplementation, a potent antixoxidant, improved quadriceps endurance in patients with COPD.[31]

4. Treating peripheral muscle dysfunction in COPD

The best available treatment option for peripheral muscle dysfunction is exercise training. Although the most commonly employed training strategy is aerobic exercises, muscle strengthening exercises are gaining popularity.[32-34] For instance, a combination of aerobic and strength training has been shown to increase mid-thigh muscle area and muscle strength in COPD.[32] The aerobic capacity of the quadriceps has been shown to improve following a 12-week training program which included 3 weekly 30-40 min exercise sessions.[35] Other benefits of training include a slower rise in blood lactate during exercise[36,37] and a reduced susceptibility to fatigue.[38]

In addition to treating peripheral muscles, exercise training may also alleviate the central component of exercise intolerance in COPD. In fact, a reduced ventilatory requirement at a given exercise level is one of the first reported evidence of physiological adaptation following exercise training in COPD.[36] As a result, exercise-induced dynamic hyperinflation is diminished[39,40], an important consideration given its negative impact on exercise tolerance in COPD.[6,12] Reduced dynamic hyperinflation during exercise is one example of how pharmacotherapy and rehabilitation can positively interact to increase functional status in COPD.

Another example of the synergistic interactions between pharmacotherapy and rehabilitation was recently provided in a study looking at the interaction between these two treatment modalities.[41]. This clinical trial is useful to illustrate the concept of synergy between drug and non drug interventions. Patients with COPD were randomized to receive tiotropium or placebo before undertaking an 8-week exercise training program (Figure 4). Although exercise endurance improved slightly with the drug alone, it is only with the combination of tiotropium and exercise training that the full advantage in terms of exercise tolerance became evident. Patients allocated to the combined treatment group had a larger improvement in exercise endurance during the training program compared to the group randomized to exercise training alone. Although the mechanisms explaining the synergistic interaction between tiotropium and exercise train-

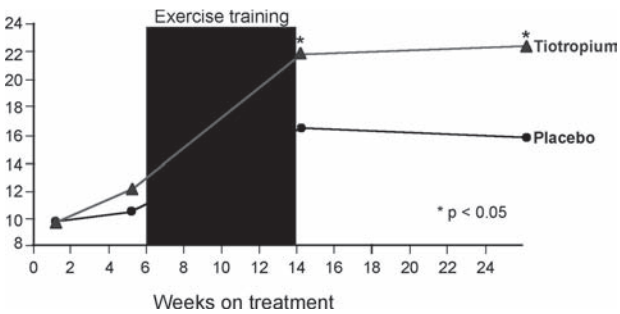

FIGURE 4. The positive interaction between bronchodilation and exercise training. From Casaburi et al. Chest 05;127:809-17.

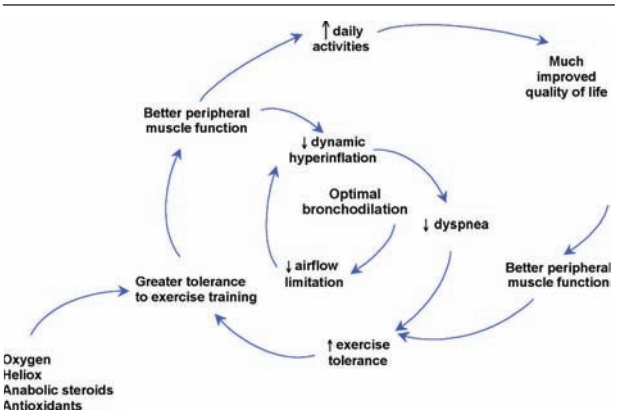

FIGURE 5. Synergistic interactions between pharmacotherapy and non-pharmacological treatement in patients with COPD.

ing where not addressed in that investigation, one plausible explanation is that optimal bronchodilation enhanced the tolerance to higher training intensities therefore leading to a better outcome after rehabilitation. In turn, patients may be enabled to take full advantage of bronchodilation with exercise training which reduces susceptibility to muscle fatigue[38] therefore minimizing its negative impact on the exercise response to bronchodilator.[16,18] Patients often gain confidence about their physical capacity during the rehabilitation program, allowing the physiological improvements seen in the laboratory to translate into better daily living and quality of life.

5. How to optimize the benefits of pulmonary rehabilitation

The magnitude of physiological improvement after exercise training is related to the intensity of training achieved, high intensity training resulting in greater physiological benefits compared to low intensity training.[36] However, this training objective is unrealistic for many patients showing poor tolerance to high training intensity. For instance, severe dyspnea during the training sessions may prevent patients from reaching higher levels of training intensities. Even though training at high intensity is not a mandatory requirement to a successful rehabilitation program, researchers are currently investigating various approaches to allow patients to attain higher exercise intensities in the hope of better clinical outcomes. Oxygen supplementation[42], heliox[43] and non-invasive ventilation[44] have all been proposed as useful adjuncts to exercise training. By decreasing exercise-induced dynamic hyperinflation[39,40,43], reducing the load on respiratory muscles[44] and alleviating shortness of breath, these approaches should allow higher exercise training intensities to be completed during the exercise sessions. Further work is nevertheless necessary to better understand the long term implications of these adjuncts to exercise training on functional status and quality of life.

The treatment of muscle wasting should be an important objective of the treatment of patients with COPD and it is possible to induce muscle growth with appropriate training stimulus.[32] This is another area where pharmacotherapy could eventually be used in conjunction with exercise training to further promote muscle growth. In a recent study, combining strengthening exercises to high dose of anabolic steroids in men with COPD and low testosterone levels was associated with a striking gain of 3.3 Kg in limb muscle mass over a 12-wk period.[45] The relevance of this muscle growth in terms of functional status or survival as well as the safety of this intervention will need to be evaluated in longer term studies. The association between muscle mass and survival[46,47] nevertheless suggests that reversing muscle atrophy should have positive prognostic implications. Other substances enhancing anabolism such as specific nutriments and antioxidants[31] are likely to be tested in association to exercise training. The near future should witness interesting development in this area.

Conclusion

Once viewed as an irreversible condition, COPD is now considered as a treatable disease. It is also clear that exercise intolerance in this disease is complex and multifactorial. This implies that, in order to reach optimal status, an array of interventions will be necessary. Treating skeletal muscle dysfunction should be an important goal and pulmonary rehabilitation is the best currently available therapy in this regard. The rationale of combining pharmacological and non-pharmacological therapies is strong and the multiple levels of synergetic interactions between these two approaches are illustrated in figure 5. By acting in a complementary and synergistic fashion, pharmacotherapy and exercise training offer the best hope for an optimal status in COPD.

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SNAPSHOT FROM THE 2004 NATIONAL PHYSICIAN SURVEY

Age of Entry to Family Practice and Specialist Residency Programs

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The median age for second year residents in both family medicine (FM; n=227) and specialty (SP; n=371) residency programs is 29 years, while the median number of years of post-secondary education prior to entering medical school is 4.0 years for both FM (n=209) and SP residents (n=335). When asked about degrees completed prior to entering medical school, 13.9% had either a Masters and/or Doctorate degree, while an additional 4.5% had a degree other than their Bachelor's or CEGEP degree.

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