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REVIEW

Measurement of body composition as a surrogate evaluation of energy balance in obese patients

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Abstract

In clinical practice obesity is primarily diagnosed through the body mass index. In order to characterize patients affected by obesity the use of traditional anthropometric measures appears misleading. Beyond the body mass index, there are overwhelming evidences towards the relevance of a more detailed description of the individual phenotype by characterizing the main body components as free-fat mass, muscle mass, and fat mass. Among the numerous techniques actually available, bioelectrical impedance analysis seems to be the most suitable in a clinical setting because it is simple, inexpensive, noninvasive, and highly reproducible. To date, there is no consensus concerning the use of one preferred equation for the resting energy expenditure in overweight and/or obese population. Energy restriction alone is an effective strategy to achieve an early and significant weight loss, however it results in a reduction of both fat and lean mass therefore promoting or aggravating an unfavourable body composition (as sarcobesity) in terms of mortality and comorbidities. Therefore the implementation of daily levels of physical activity should be simultaneously promoted. The major role of muscle mass in the energy balance has been recently established by the rising prevalence of the combination of two condition as sarcopenia and obesity. Physical exercise stimulates energy expenditure, thereby directly improving energy balance, and also promotes adaptations such as fiber type, mitochondrial biogenesis, improvement of insulin resistance, and release of myokines, which may influence different tissues, including muscle.

Key words: Obesity; Body composition; Bioelectrical impedance analysis; Energy expenditure; Sarcobesity

Core tip: There are overwhelming evidences towards the relevance of a more detailed description of the individual phenotype by characterizing the main body components as free-fat mass, muscle mass, and fat mass. Among the numerous techniques actually available, bioelectrical impedance analysis seems to be the most suitable in a clinical setting because it is simple, inexpensive, noninvasive, and highly reproducible. To date, there is no consensus concerning the use of one preferred equation for the resting energy expenditure in overweight and/or obese population.

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INTRODUCTION

Obesity, defined by excessive adipose tissue, has been shown several deleterious effects on many body organs through thrombogenic, atherogenic, oncogenic, hemodynamic and neurohumoral pathways and has been linked to several chronic diseases, as diabetes, ischemic heart, and musculo-skeletal disorders, together with malignancies. Overweight and obesity represent the fifth leading risk for global death by The World Health Organization on March 2013[1]. At least 2.8 million adults die each year as a result of overweight/obesity. Moreover the 44% of diabetes and 23% of ischemic heart disease burden can be attributable to an abnormal adipose tissue accumulation[1].

Obesity is diagnosed accordingly to the body mass index (BMI). This index has been strongly recommended for use in clinical practice[2,3]. Multiple studies have shown a U or J-shaped relation between BMI, all causes and/or cardiovascular mortality, thus identifying the best survival rate for BMI values in overweight (25-27 kg/m^2) followed by a dramatic increase in risk profile out of these values[4-9].

In a cross-sectional study enrolling 13601 subjects (45.5 ± 17 years; 48% men) from the Third National Health and Nutrition Examination Survey (NHANES)[10], the BMI cut-off of $\geqslant$ 30 kg/m^2 shown an overall low sensitivity and an high specificity to identify obesity such as defined by an excessive body fat percentage at bioelectrical impedance analysis (> 25% in men and > 35% in women, respectively, thus according to the gold standard definition proposed by the World Health Organization[2]. The BMI-based definition of obesity has important limitations on diagnostic performance, particularly in men and elderly, missing more than 50% of people with excessive fat mass. In men, BMI showed a more reliable association with lean mass than with total body fat. In contrast, in women BMI appears to be more accurate to estimate fat-mass, thus explaining why overweight women have been reported as more consistently related to increased mortality compared to overweight male individuals. Furthermore, there is significant inter-subject variability in body fat percentage in individuals with similar BMI values. About 30% of obese subjects do not show any metabolic complications such as diabetes, hypertension, dyslipidemia, *etc*.), then they can be defined metabolically healthy obese patients[11]. On the other hand, in subgroups of BMI-based normal weight individuals an increased cardiometabolic risk profile and an insulin resistant condition could be described and strictly related to fat mass accumulation[12,13].

Further studies have pointed out the association between visceral adiposity, better than BMI, mortality and cardiovascular disease. Waist circumference as a marker of abdominal adipose tissue, has been placed as one of the main contributors to the Metabolic Syndrome by the National Cholesterol Education Program Adult Treatment Panel Ⅲ in 2001[14], then as the core feature of the diagnostic criteria proposed by the International Diabetes Federation in 2005.

In the International Day for Evaluation of Abdominal adiposity cross-sectional study, enrolling 168.000 patients in 63 countries, waist circumference showed a higher adjusted odds ratio (95%CI) for diabetes than BMI in overall sample population [1.35 (1.30-1.40) *vs* 1.23 (1.19-1.27) for men and 1.55 (1.50-1.60) *vs* 1.23 (1.19-1.27) for women, respectively) such as for cardiovascular disease [1.24 (1.19-1.28) *vs* 1.13 (1.09-1.17) for men and 1.21 (1.17-1.25) *vs* 1.20 (1.16-1.24) for women, respectively)[15].

In the NHANES Ⅰ and NHANES Ⅱ longitudinal prospective cohort studies, performed in 10169 male subjects a continuous positive relationship between fat-mass and all-cause mortality rate (HR = 1.033, 95%CI: 1.005-1.063, P = 0.0213) have been reported, together with a preponderant significantly negative relationship between free-fat mass and all-cause mortality (*i.e.*, protective; HR = 0.923, 95%CI: 0.906-0.941, P < 0.0001)[16].

Furthermore, BMI and weight loss rate may not be linked to clinical improvement of health-related outcomes, in comparison to different body composition measures by simple and non-invasive methods, which demonstrated a strong relationship between mortality, body lean mass and adipose tissue[17-24].

In conclusion beyond the BMI, there are overwhelming evidences towards a more detailed description of the individual phenotype by characterizing the main body components as free-fat mass (FFM), muscle mass (MM), and fat mass (FM).

BODY COMPOSITION MEASUREMENTS: TECHNOLOGICAL ADVANCES AND CURRENTLY AVAILABLE NON INVASIVE TECHNIQUES

Numerous techniques for body composition analysis are currently available: anthropometry, including the 4-skinfold method, hydrostatic weighing, *in vivo* neutron activation analysis, anthropogammametry from total body ^{40}K, nuclear magnetic resonance, dual-energy X-ray absorptiometry (DEXA), computerized tomography (CT), and bioelectrical impedance analysis (BIA). Evaluating complexity, invasiveness, and cost only DEXA, CT, and BIA may represent the methods of choice to assess body composition in clinical practice whereas the other techniques are limited to scientific

purposes.

DEXA estimates body fat and lean mass percentage through a tissue-specific model (fat, lean tissues, and bone) based on X-ray (dose 1-3 mrad) tissue-dependent attenuation[25]. The largest sample (*n* = 22000 partecipants) have been analyzed by the NHANES[26]. DEXA systems currently available for scanning whole-body tissue composition are capable to analyze a wide range of weights including severe obese subjects (> 150 kg). DEXA scans can be subdivided into different body regions, *i.e.*, trunk, arms, and legs, thus identifying and estimating both android and gynoid fat distribution. DEXA can detect only abdominal adipose tissue accumulation without any distinction between visceral and subcutaneous fat because of their similar X-ray attenuation properties and tissue overlapping[27]. DEXA is the gold standard technique for the evaluation of body composition in clinical research[28-30], although limited in clinical practice by the radiation exposure, availability and cost. The use of the same DEXA instruments and analysis software are of relevant importance for longitudinal studies, since they could influence body composition measurements[31].

In patients affected by malignancies, the analysis at the level of the 3rd lumbar vertebra by CT strongly predicted whole body fat and FFM as compared with DEXA[32]. CT provided an accurate evaluation of body composition, not provided by DEXA or BIA and a X-ray exposition similar to a chest radiography. Although not validated, also CT images of the right thigh halfway showed to be significantly related to overall mortality rate in chronic obstructive pulmonary disease patients[33]. Despite its wide use at diagnosis and follow-up in severely ill patients, CT scan at the 3rd lumbar vertebra cannot be considered a feasible method to assess body composition in obese population requiring expensive equipment, trained operators and exposure to ionizing radiation.

BIA is based on the capacity of hydrated tissues to conduct electrical energy. The BIA methodology have been described in two ESPEN position papers[34,35]. The analysis of total body impedance is based on the estimation of total body water. From total body water, prediction equations allow the calculation of FFM and FM. BIA equations have been validated for chronic obstructive pulmonary disease, AIDS, transplant patients and elderly individuals[36]. A prediction equation for FFM estimation by BIA in adults (age: 20-94 years; BMI: 17.0-33.8 kg/m^2) has been proposed[37] as well as reference values of FFM and FM for a Caucasian population[38]. Previously reported data showed no significant differences between DEXA and BIA for the assessment of fat mass in overweight and/or obese patients with a significantly good linear correlation[39,40]. However several factors could limit the validity of BIA in severe obesity[41].

ENERGY BALANCE IMPLICATIONS FOR ADDRESSING OBESITY: AVAILABLE ENERGY EXPENDITURE MEASUREMENTS

Energy balance is constituted by three major components including energy intake, energy expenditure (EE) and energy storage. Energy expenditure is expressed by resting energy expenditure (REE), as the amount of energy required for the endogenous metabolic activity separated from the metabolic effects of food and physical activity, the food-related thermic effects (TEF), as energy need to absorb and metabolize ingested food, and energy expenditure associated to physical activity (EE_{PA}). EE_{PA} is the most inter-individual variable component of energy expenditure and consists of the amount of physical activity performed multiplied the energy requirement of that activity. The REE is proportional to body mass, particularly to the FFM[42]. The REE prediction equations evaluate the energy expenditure of 2 different body compartments as the adipose tissue or FM and the lean mass or FFM. FFM is the main source for REE and is commonly considered as a surrogate measure of metabolically active tissues. Brain, liver, heart, and kidney account for approximately the 60% of REE, despite a combined weight < 7% of FFM. In comparison to the skeletal muscle, the metabolic rate of heart and kidney is approximately 30-fold higher, and the later approximately 2-fold higher in comparison to brain and liver. The skeletal muscle represents the major contributor to the FFM (50%), but accounts for only the 21% of the REE. Adipose tissue has a low energy expenditure, however its body rate varies significantly according to the dramatic increase of overweight and obesity. Then it is notable that FM represent an important source for the REE in all prediction models. Among adults, REE is lower in elderly, almost in part explained by the change in body composition[27].

Advantage and limitations of currently available non invasive techniques for the estimation of EE are summarized in Table 1.

Indirect calorimetry represents the gold standard technique to evaluate REE. This method used the rates of oxygen consumption (V_{O2}) and/or carbon dioxide production (V_{CO2}) to calculate EE according to the Weir predictive equation, derived from studies comparing indirect calorimetry with direct calorimetry[43]. The complexity and cost, together with the requirement of the patient isolation for at least 24 h limit the feasibility of the direct calorimetry in humans.

The indirect calorimetry consists in a gas collector, a canopy and a system that measures the volume and concentrations of O_2 and CO_2 minute by minute. Through a unidirectional valve located in the ventilated canopy, the calorimeter collect and quantify the volume and concentration of O_2 inspired and of CO_2 expired. A systematic literature review aimed to determine the

Table 1 Advantages and limitations of available techniques the measurement of Energy Expenditure

Technique	Advantages	Limitations
Direct calorimetry	The gold standard method for measure EE in animal models	High complexity, high cost, need to confine the subject for almost 24 h
Indirect calorimetry	The gold standard to measure REE in humans. Non invasive, adequately accurate, highly reproducible	High cost, relatively complex, need of trained personnel
Bioelectrical impedance analysis	Non invasive, simple, adequately accurate for body composition analysis, relatively inexpensive	The estimation of EE is limited by the need of obesity-specific predictive equations
Multi-sensor device	Easy and practical to use	The estimation of EE is limited by the need of obesity-specific predictive equation

EE: Energy expenditure; REE: Resting energy expenditure.

optimal conditions for obtaining reliable measures of REE by indirect calorimetry recommends fasting for at least 6 h, avoiding caffeine during the night, nicotine and alcohol for at least 2 h, moderate physical activity for at least 2 h, and vigorous physical activity for 14 h before[44]. Despite methodological, environmental and individual limitations indirect calorimetry represents a non-invasive and very accurate method to estimate REE[45]. However this method is widely limited in most of clinical settings by the requirement of expensive equipment, and trained operators, then many efforts are spent to identify the most accurate predictive equation to determine REE in overweight and obesity.

Predictive equations have generally been calculated from experimental studies performed in healthy subjects on the basis of regression analysis of bodyweight, height, sex, and age as independent variables and REE by indirect calorimetry as a dependent variable. On the basis of a review of available evidences from Harris and Benedict[46], FAO/WHO/UNU weight or weight and height equations[47], and the equations of Mifflin *et al*[48] and Owen *et al*[49], Frankenfield *et al*[50] proposed the use of the Mifflin equation both for overweight and obese subjects. All the available predictive equations have been further validated with indirect calorimetry from adults aged 18-65 years with a BMI of 25 to 40 kg/m^2 in order to identify the most accurate and precise REE predictive equation in specific overweight and obese groups of United States and Dutch subjects: the results of this study were similar to the data of Frankenfield *et al*[50], then supporting the use of Mifflin equation in the United States. However for overweight and obese Dutch adults, there appears to be no accurate equation[51]. This discrepancy for the Dutch than for the United States adults could be explained by the difference about weight and height values, even within sex and BMI subgroups, thus limiting the validity of this equation in similar taller populations. Numerous studies contributed to further evaluate the currently available predictive equations in overweight and obese subjects[52-55] and/or in extremely obese subjects[56-61]. There is some evidences supporting the Mifflin[59], the FAOw[56,58] and the Harris Benedict[60], the Siervo equations[61] in extremely obese subjects. More recently a new equation, using FFM, Horie-Waitzberg, and Gonzalez equations, have been validated and proposed in Brazilian severely obese subjects[62]. The most commonly proposed equation for the measurement of REE have reported in Table 2.

To date there is no consensus about the use of one REE equation compared to others, in overweight and/or obese population. This might be related to differences about group composition, methods, or statistical analysis, at least in part.

In order to estimate EE including EE_{PA}, a practical multi-sensor device, the SenseWear Armband (BodyMedia, Inc., Pittsburgh) has been recently developed. This device contains four sensors to detect heat flux, accelerometry, galvanic skin reaction, skin temperature. Despite excellent results by comparison with energy expenditure measured using the doubly labeled water in overweight/obese children[63] and lactating women[64], other evidences underline the need of obesity-specific equations[65,66].

A meta-analysis of randomized clinical trial suggest that pedometer use is associated with a significant decrease of body weight and blood pressure[67]. However a recent review assessing different accelerometers to measure daily physical activity as a surrogate of EE in comparison with the doubly labeled water only few available devices have been proven to adequately correlate with the reference method[68].

BEYOND THE ADIPOSE TISSUE EXCESS: THE IMPORTANCE OF MUSCLE MASS IN OBESITY

Skeletal muscle plays a critical role in the glucose metabolism and peripheral insulin sensitivity as well as musculoskeletal performance. The importance of lean mass on energy balance has been widely recognized by the rising prevalence of the combination of two condition as sarcopenia and obesity[69]. Two major consensus documents provide different definition of sarcopenia. The European consensus, the Working Group on Sarcopenia in Older People, defines sarcopenia as generalized loss of skeletal muscle mass and strength[70]. The International consensus, the International Working Group on Sarcopenia, requires a decline in muscle mass and walking speed to diagnose sarcopenia[71]. Although sarcopenic obesity is commonly used to define the coexistence of diminished muscle mass and increased adipose tissue, a standard

Table 2 Most commonly proposed predictive equations for the estimation of the resting energy expenditure

	Age	Sex	Equation
Harris and Benedict (kcal/d)	15-74	Male	66.4730 + 13.7516 (W) + 5.0033 (H) – 6.7550 (A)
	15-74	Female	655.0955 + 9.5634 (W) + 1.8496 (H) – 4.6756 (A)
Schofield (MJ/die)	10-17	Male	0.074 (W) + 2.754
	10-17	Female	0.056 (W) + 2.898
	18-29	Male	0.063 (W) + 2.896
	18-29	Female	0.062 (W) + 2.036
	30-59	Male	0.048 (W) + 3.653
	30-59	Female	0.034 (W) + 3.538
	⩾ 60	Male	0.049 (W) + 2.459
	⩾ 60	Female	0.038 (W) + 2.755
FAO/ WHO/ UNU (MJ/d)	10-17	Male	0.0732 (W) + 2.72
	10-17	Female	0.0510 (W) + 3.12
	18-29	Male	0.0640 (W) + 2.84
	18-29	Female	0.0615 (W) + 2.08
	30-60	Male	0.0485 (W) + 3.67
	30-60	Female	0.0364 (W) + 3.47
	> 60	Male	0.0565 (W) + 2.04
	> 60	Female	0.0439 (W) + 2.49
Mifflin-St Jeor (kcal/d)	19-78	Male	10 × W + 6.25 × H – 5 × A + 5
	19-78	Female	10 × W + 6.25 × H – 5 × A – 161
Owen (kcal/d)	18-65	Male	879 + 10.2 × W
	18-65	Female	795 + 7.18 × W

To convert MJ into kcal, multiply the result by 239. W: Body weight (kg); H: Height (cm); A: Age (years).

definition of sarcopenic obesity is still lacking. Several clinical studies have indicated that obesity and/or insulin resistance may underlie the development of sarcopenia[72]. A possible role of vitamin D in sarcopenia has been postulated in two studies demonstrating that serum 25-hydroxy vitamin D was negatively correlated with appendicular (legs and arms) fat mass and positively associated with appendicular muscle mass, both evaluated through DEXA analysis[73,74].

Fat mass excess may induce and/or worsen sarcopenia because the increase of lipid depot reduces both amino acid utilization and protein synthesis in muscle fibers. Evaluating 3132 elderly male subjects without diabetes the highest quartile of homeostasis model assessment of insulin resistance showed the highest risk for a decrease in lean body mass and appendicular mass[75]. On the counterpart, skeletal muscle is the main target of insulin, then loss of muscle mass may cause insulin resistance. A previous study identified sarcopenia as a risk factor for exacerbating insulin resistance in obese subject with dysglycemia[76].

Skeletal muscle fibers can be classified into different categories: from slow (type Ⅰ) fibers with low contractile abilities, numerous mitochondria and high oxidative energy metabolism, to type Ⅱa and type Ⅱd/x, and eventually fast (type Ⅱb) fibers able to contract rapidly and predisposed toward glycolytic processes. In obese and diabetic population skeletal muscle commonly contains more type Ⅱb fibers, while slow muscle fiber percentage and skeletal muscle glucose transport are significantly reduced. Sarcopenic individuals have been shown to be associated with a further impairment of muscle fiber content, predominantly of type Ⅰ [77]. Recent studies have shown that exercise is able to increase the content of type Ⅰ and type Ⅱa fibers[78]. Furthermore, in skeletal muscle with ageing both the number and morphology of mitochondria are changed with an impaired function and an associated reduced oxidative capacity[79]. In particular since the primary role of mitochondria is to maintain the cellular energy balance, shifts in mitochondria respiratory activity can lead to a reduced maximal capacity of the tricarboxylic acid cycle and the electron transport chain together with an incomplete lipid-induced upregulation of β-oxidation rates, thus inducing a significant accumulation of intramyocellular lipids and further impairing whole body insulin resistance[79]. Physical exercise has been demonstrated to induce mitochondria biogenesis, upregulates skeletal muscle gene expression and protein synthesis, and increases skeletal muscle oxidative capacity both in older and sedentary populations[80].

Moreover, recent evidences promote the hypothesis of cross talk between muscle and different tissues mediated by cytokines and other peptides called myokines[81] which are involved both in acute exercise-induced metabolic reactions, as well as in the long-term metabolic benefits induced by exercise[82]. Irisin is one of the most recently identified myokines. Following regular physical activity, Irisin increases two-fold its circulating levels and promotes the shift of white adipocytes into "brite" cells: white adipocytes with a brown-fat-like phenotype. Brown adipocytes activate thermogenesis *via* the mitochondria uncoupling protein UCP-1 (Figure 1). Overexpression of irisin

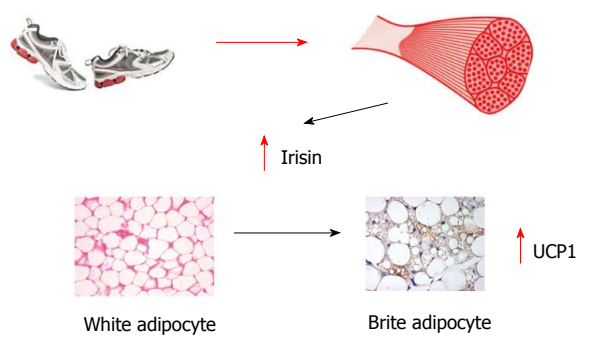

Figure 1 Physical activity increases the intramuscular expression of the membrane protein fibronectin type III domain containing 5 (FNDC5) which is cleaved to form Irisin, a myokin. Irisin may stimulate the transformation of white adipocytes into brite cells, thus suggested by a marked increase in the expression of uncoupling protein 1 (UCP-1). Adapted from Pedersen[82].

determined through a gene therapy approach mediated by adenoviral particles, and leading to a modest approximately 3 fold increase in circulating levels, induced browning of subcutaneous white adipose tissue, stimulating a 10-20 fold increase in UCP1, thus increasing energy expenditure and improving glucose tolerance of high fat fed mice[83]. Despite more recent *in vitro* evidences against the possible translation of the beneficial effects observed in mice to humans, the major role of muscle mass in the energy balance has been definitively established.

In conclusion, physical exercise increases EE and stimulates muscle adaptation mechanisms with potential benefits on different tissues.

ENERGY BALANCE, PHYSICAL ACTIVITY AND DIETARY RESTRICTION

Analysis from the NHANES database describes an average daily intake increase of 168 kcal/d for men and of 335 kcal/d for women, from 1971 to 2000[84]. On the counterpart, examining in 2004, the physical activity patterns in a typical agrarian population in the United States through pedometers an average of about 18000 steps per day in men and of about 14000 steps per day have been reported[85], whereas an average American adult walked about 5000 steps per day[86].

An energy balance flipping point between energy intake and energy expenditure could be recognized in United States around 60' years, thereafter followed by energy intake continuously driving energy expenditure, together with a worldwide burden in the incidence of obesity and type 2 diabetes[87].

The most frequently proposed strategy for treating obesity is food restriction. Energy restriction alone is an effective strategy to achieve a rapid and substantial weight loss, however it results in a reduction of both fat and muscle mass therefore promoting or aggravating an detrimental body composition (as sarcobesity) in terms of mortality and comorbidities. Specifically, approximately 25% of weight loss obtained through short-term low energy diets is lean muscle mass[88-92]. Moreover, the lack of success in weight loss maintenance after low-energy regimen and the subsequent weight regained comprising of up to 80% fat mass compound a further detrimental body composition impairment[93,94]. Weight loss should not be considered the sole focus of therapeutic approach aimed to decrease obesity-related disease risk profile. A systematic review by Chaston *et al*[95] demonstrates that very low calories regimens (VLCD) result in significant greater loss of FFM (lean) in comparison to low-calories diets[95]. Current evidences sustained that the most beneficial long-term outcomes are achieved with a modest energy deficit (2000-4000 kJ/d)[96]. Therefore increased levels of daily physical activity should be simultaneously promoted. Although resistance training retains or improves the relative percentage of lean mass to total body, current evidence failed to detect a loss of fat mass of a similar magnitude to aerobic training which promotes fat mass loss together with beneficial changes in muscle tissue[97-99].

Studies evaluating efficacy of dietary restriction together with exercise program showed more favorable results (as weight loss rate and body composition improvement) compared to diet or exercise prescription alone[69]. Emerging data suggest that the combination of resistance and aerobic exercise with a modest energy restriction was successful for preserving skeletal muscle concomitantly with a significant decrease of fat mass[100].

CONCLUSION

In order to characterize patients affected by obesity the use of traditional anthropometric measures appears misleading. A systematic and deep phenotyping of these patients, thus integrating data from body composition analysis and energy expenditure should be used in a dynamic rather than only basal approach to define and periodically verify the efficacy of the therapeutic regimen proposed. Among the numerous techniques evaluated in this paper, BIA seems to be the most suitable in a clinical setting because it is simple, inexpensive, noninvasive, and highly reproducible. Moreover, a recent paper by our research group showed that the FM and MM percentage estimated by BIA at baseline should be considered as predictors of success (weight loss > 5% at 6 mo from baseline) in a individual cognitive-behavioral program[40].

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EDITORIAL

Ability of community-based prostate cancer screening to target an appropriate and underserved population

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Abstract

Screening is not universally beneficial due to over- and under-diagnosis, and false positives that beget additional testing and associated adverse events and expense. We examined data from all men who participated in a mass community prostate cancer screening between May 2009 and September 2010. The data contained information regarding patient demographics, family history of prostate cancer, lower urinary tract symptoms, prior history of prostate cancer, most recent digital rectal examination, and the presence of an established relationship with a physician. Current American Urological Association screening recommendations were then applied to determine the appropriateness of our outreach effort. A total of 438 men (mean age 66.5 years) underwent screening. A total of 106 (24.2%) patients in our study met contemporary criteria for screening. Of these men, the vast majority was well educated, well insured, and well informed about the need for prostate cancer screening. Based on these data, mass community-based prostate cancer screening does not appear to identify and screen at-risk men. Future efforts at mass screening should more carefully target men most likely to benefit.

Key words: Prostate cancer; Screening; Outcomes; Prostate specific antigen; Community health

Core tip: Mass prostate specific antigen-based prostate screening is used throughout the world as a means of reducing prostate cancer morbidity and mortality. However, a large proportion of men who underwent mass screening in our region were, in hindsight, not appropriate candidates for screening. Given the recent warnings of the United States Preventative Services Task Force and American Urological Association regarding the over-diagnosis of prostate cancer, it is incumbent on urologists, hospitals, and public health agencies to critically examine the role of screening practices, recognizing both the potential for community benefit

and of harm from inappropriate screening.

Moss J, Heidel E, Johnson J, Powell C, Dittrich E, Rawn S, Terry PD, Goldman M, Waters WB, White WM. Ability of community-based prostate cancer screening to target an appropriate and underserved population. *World J Methodol* 2015; 5(2): 10-12 Available from: URL: http://www.wjgnet.com/2222-0682/full/v5/i2/10.htm DOI: http://dx.doi.org/10.5662/wjm.v5.i2.10

INTRODUCTION

Prostate cancer is the second most common malignancy diagnosed in American men with an annual estimated incidence of approximately 240000[1]. The introduction of prostate specific antigen (PSA) screening has effected a stage migration that has led to earlier diagnoses and the perception of improved survival[2]. Recently, the United States Preventative Services Task Force suggested that PSA-based screening is unnecessary and potentially harmful in some groups of men[3]. Consistent with the Task Force, the American Urological Association (AUA) currently discourages the common practice of "mass" screening[4].

OUR EXPERIENCE WITH MASS PROSTATE CANCER SCREENING

Our institution has previously offered PSA-based prostate screening to our community without adherence to any specific guidelines. Therefore, we retrospectively examined the nature of our prior screenings to determine if our outreach efforts were targeting appropriate screening candidates and/or an underserved population.

Prostate cancer screening was offered to all men in the Knoxville, Tennessee metropolitan area. Through mass mailings, social media, and traditional media formats, men were invited to one of nearly 10 geographically distributed screening locations. All participants provided information regarding demographics, baseline prostate health, family history of prostate cancer, prior screening, and access to either an urologist or primary care physician. Participants then underwent PSA testing and a digital rectal examination (DRE) by a board certified urologist. Results of the DRE were categorized as normal, abnormal concerning or highly suspicious for cancer, or enlarged consistent with benign prostatic hyperplasia. PSA values were forwarded to the screening provider and compared to examination results, and the patient was either (1) advised to undergo routine screening once a year; or (2) encouraged to follow-up on his "abnormal results" for additional confirmatory testing.

We retrospectively examined demographic data from these patient-reported forms, called patients individually to confirm accuracy of these results, and applied current best practice screening guidelines based on AUA recommendations[4]. Men aged 55-69 years were considered appropriate screening candidates, whereas men outside of this age range and/or those who had undergone prior screening within one year and/or men with a prior diagnosis of prostate cancer were considered poor screening candidates. African-American men and/or men with a family history of prostate cancer within a first-degree relative were considered appropriate candidates for screening. The Statistical Package for the Social Sciences version 21 (Armonk, NY: IBM Corp) was used to calculate frequency and cross-tabulation statistics to assess characteristics of the dataset. The University of Tennessee Graduate School of Medicine Institutional Review Board approved the study.

Between May 1, 2009 and September 30, 2010, 438 men underwent PSA-based prostate cancer screening. The mean age of the cohort was 66.5 years (age range 37-91). In this cohort, 98% were Caucasian, 16% reported family history of prostate cancer, and 27.1% reported attendance at a similar screening event within the past 12 mo. In addition, 97.6% and 95.3% reported an understanding of the need for annual PSA and DRE, respectively. Two-thirds of screened individuals had completed some form of higher education (greater than a high school degree, reflecting a better-educated population than the more general regional population. Approximately 95% of the cohort maintained health insurance at the time of screening. In all, 87.3% of the total cohort reported an established relationship with a primary care physician. Finally, nearly 97% were educated about the need for an annual PSA and DRE, and at least 80% of patients had attended mass screening at some point in the past. Taken as a whole, men participating in our mass prostate cancer screening represent a well educated, insured population; relatively few of those men were deemed to be appropriate for screening.

DISCUSSION

We found that a large proportion of men who underwent screening in our cohort were not appropriate candidates for screening. Further, most of the men who "met criteria" for screening based on age, ethnicity, and/or family history, were well educated, well insured, and well informed; hence, men most in need of screening were largely absent from our mass screening effort.

Screening is not universally beneficial due to the overdiagnosis of potentially indolent disease, false positives that beget additional testing and associated adverse events and expense, and poor sensitivity that may lead to underdiagnosis and a false sense of security. Given the criticism centered on PSA-based screening and "mass screening" in particular, it is incumbent on urologists, hospitals, and public health agencies to critically examine the role of screening

practices, recognizing both the potential for community benefit and of harm from inappropriate screening.

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EDITORIAL

Recurrent urinary tract infections in children: Preventive interventions other than prophylactic antibiotics

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Abstract

Urinary tract infection (UTI) is one of the most common childhood infections. Permanent renal cortical scarring may occur in affected children, especially with recurrent UTIs, leading to long-term complications such as hypertension and chronic renal failure. To prevent such damage, several interventions to prevent UTI recurrences have been tried. The most established and accepted prevention at present is low dose long-term antibiotic prophylaxis. However it has a risk of break through infections, adverse drug reactions and also the risk of developing antibiotic resistance. The search is therefore on-going to find a safer, effective and acceptable alternative. A recent meta-analysis did not support routine circumcision for normal boys with no risk factors. Vaccinium Macrocarpon (cranberry), commonly used against UTI in adult women, is also effective in reducing the number of recurrences and related antimicrobial use in children. Sodium pentosanpolysulfate, which prevents bacterial adherence to the uroepithelial cells in animal models, has shown conflicting results in human trials. When combined with antibiotic, *Lactobacillus acidophilus* (LA-5) and *Bifidobacterium*, by blocking the *in vitro* attachment of uropathogenic bacteria to uroepithelial cells, significantly reduce in the incidence of febrile UTIs. Deliberate colonization of the human urinary tract of patients with recurrent UTI with *Escherichia-coli* (*E. coli*) 83972 has resulted in subjective benefit and less UTI requiring treatment. The non-pathogenic *E. coli* isolate NU14 DeltawaaL is a candidate to develop live-attenuated vaccine for the treatment and prevention of acute and recurrent UTI. Diagnosing and treating dysfunctional elimination syndromes decrease the incidence of recurrent UTI. A meta-analysis found the lack of robust prospective randomized controlled trials limited the strength of the established guidelines for surgical management of vesicoureteral reflux. In conclusion, several interventions, other than antibiotic prophylaxis, for the prevention of recurrent UTI have been tried and, although showing some promise, they do not provide so far a definitive effective answer. Finding suitable alternatives still requires further high quality research of those seemingly promising interventions.

Key words: Kidney; Recurrence/prevention; Urinary tract infections; Vesico-ureteral reflux; Vaccinium macrocarpon; Circumcision; Vaccination; Constipation; Lactobacillus

Core tip: Antibiotic prophylaxis against urinary tract infection recurrences is associated with adverse drug reaction and development of resistance. Although showing some promise, alternative interventions, such as Vaccinium Macrocarpon (cranberry), *Lactobacillus* and Probiotics, circumcision, surgical management of vesicoureteral reflux, deliberate colonization of the urinary tract with *Escherichia-coli* (*E. coli*) 83972, treating constipation and dysfunctional voiding, administration of synthetic substitutes that reproduce natural surface glycosaminoglycan(s) anti-adherence effect on uroepithelial cells and *E. coli* isolate NU14 DeltawaaL as a candidate for developing a live-attenuated vaccine, do not provide so far a definitive effective answer. Further high quality research is still required.

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INTRODUCTION

Urinary tract Infection (UTI) is one of the most common childhood infections[1,2], affecting round 1.7% of boys and 8.4% of girls by the age of seven years[3]. A third to one half of affected children will suffer from at least one recurrence[4]. While in infancy this infection is mostly secondary to hematogenous dissemination, it usually occurs as an ascending infection in older children, where the common organism involved include Gram negative bacteria such as *Escherichia-coli* (*E. coli*), *Klebsiella*, *Proteus*, *Enterobacter*, *Pseudomonas* and *Serratia* species[5]. Permanent renal cortical scarring may occur in 15%-65% of affected children[4], especially in recurrent UTI and its long-term complications include hypertension and chronic renal failure which may result in end stage renal disease[6]. Contrary to previous beliefs, acquired renal scarring correlates best with recurrent UTI rather than the presence of vesicoureteral reflux (VUR). In a bid to prevent long-term damage with recurrent UTI several interventions have been tried, aiming at one or more of the factors that facilitate the development of UTI.

These factors include: (1) microbial growth in the urogenital tract; (2) foreskin facilitating peri-meatal bacterial growth; (3) bacterial adhesion to the uroepithelial cells; (4) bioflora favoring pathogenic urobacteria; (5) insufficient urothelial cytokine secretion; (6) urinary stasis; and (7) vesicoureteral reflux. The interventions considered included therefore: (1) long term antibiotic prophylaxis; (2) circumcision in male children; (3) cranberry and glycosaminoglycans; (4) lactobacillus and probiotics products; (5) vaccination; (6) management of dysfunctional elimination syndrome; and (7) vesicoureteral reimplantation[4].

PREVENTIVE INTERVENTIONS

Antibiotic prophylaxis

This is currently the most established and accepted prevention of UTI recurrences at present[7]. However, this policy is of limited efficacy with little or no clinical benefit at all in various trials[8-10]. Although compliance to treatment is reported to be 91%, only 31% of children have antibiotic metabolites identified in the urine[11]. Furthermore, the risk of break through infections is estimated to be between 25% and 38%[12,13]. Antibiotic usage is not without risk. Approximately 10% of children on long-term antibiotic therapy develop adverse reactions that range from common gastrointestinal symptoms to bone-marrow suppression and rarely Stevens Johnson Syndrome[14,15]. Also worrisome is the growing evidence of antibiotic resistance developing with excessive use of long-term antibiotics[16].

Considering all those concerns with antibiotic prophylaxis, there is a growing need to re-evaluate the other suggested alternative interventions to prevent UTI recurrences. We look at the currently available evidence behind these alternative interventions.

Circumcision

Although for a long time the absolute indications for circumcision have been phymosis secondary to xerotica obliterans and recurrent balanoposthitis, prevention of UTI in boys has been added after a brief report that showed that the foreskin was a risk factor for UTI in male infants[17]. Several studies have since supported the benefits of neonatal circumcision, especially as the complication rate was found to be only 1.6%, consisting mainly of haemorrhage, inappropriate penile appearance, ring impaction or stenosis[18]. When compared to 3000 uncircumcised newborns where the rate of UTI is 2%, no UTI was found in any of the other 3000 circumcised neonates up to 15 mo after the procedure[18]. Circumcision significantly reduced the incidence of UTI in male children by 90%[19,20], however it is limited to one particular group of sex, and the incidence of UTI in boys is only 1% of total UTI population. With previous studies suggesting that uropathogen's attachment to the foreskin, by providing a environment for bacterial colonisation, made the foreskin a risk factor for UTI, a study was conducted on children with low grade VUR and showed that, when compared to antibiotic prophylaxis alone, circumcision associated with antibiotic prophylaxis resulted in a significant decrease in bacterial colonisation rate[21]. In a cohort of infants with antenatal hydronephrosis, circumcision

provided a significant reduction in the frequency of UTI frequency when comparing the periods before and after circumcision[22].

The support for neonatal circumcision to prevent recurrent UTI is still being challenged. In a study of ritually circumcised Jewish male neonates, there was a high prevalence of UTI, suggesting that the procedure puts the infants at an increased risk of UTI[23]. It has been suggested that the differences in UTI incidence between circumcised and non-circumcised boys is not due to the procedure, but could instead be attributed to several confounding factors such as prematurity, low birth weight, perinatal anoxia, lack of breast feeding, poor hygienic practices, low parental education, prenatal maternal UTI, history of a UTI in a first degree relative, history of fever in the mother at the time of delivery, previous infections, previous course of antibiotics, method of urine collection, and diagnostic standards used[24]. Furthermore, no effect on the incidence of postoperative UTI was found with circumcision performed during anti-reflux surgery[25].

To clear the confusion, a meta-analysis of 12 studies on circumcision and UTI prevention was conducted and, although the procedure seemed to be more beneficial to boys with recurrent UTI (only 11 needed to prevent 1 UTI) and boys with grade III or more VUR (four needed to prevent one UTI), it was calculated that overall, 111 circumcisions would be required to prevent one UTI, costing £55000 in the United Kingdom. The study concluded that a decision to carry our routine circumcision for normal boys with no risk factors was not supported by that meta-analysis[26]. In addition, although, in its policy on circumcision in 1989, the American of Paediatrics concluded that newborn circumcision decreased the rate of UTI from 1% to 0.1%, it modified the guideline in 1999 stating that routine circumcision was not necessary in all newborns[27].

Preventing bacterial adhesion to the uroepithelial cells

Vaccinium macrocarpon (Cranberry): Vaccinium macrocarpon, also called large cranberry, American cranberry and bearberry, is a cranberry of the subgenus *Oxycoccus* and genus *Vaccinium*. It is one of the most commonly used and acceptable preventive agent against UTI in adult women, and has also been tried in pediatric age groups where it was associated with a much better compliance than oral antibiotics and without significant side effects[15,28].

The mechanism of action of cranberry resides in the action of proanthocyanidine it contains on mannose-resistant P-fimbriated *E. coli*, strains that cause cystitis and pyelonephritis[29]. The proanthocyanidine containing "A" type linkage prevents the adhesion of proteinaceous fibres or fimbriae [heteropolymeric fibers carrying a Gal (alpha 1-4) Gal-specific PapG adhesin at its distal end and located on the bacterial cell] to the specific carbohydrate receptors on uro-epithelial cells[30-32]. This effect occurs at a concentration as low as 75 ug/mL[33].

Data on the effectiveness of cranberry in the prevention of UTI in adults is encouraging but still incomplete. In premenopausal women, while cranberry juice did not significantly reduce UTI risk compared with placebo, the reduction in urinary P-fimbriated *E. coli* strains supported the biological plausibility of its activity[34]. In renal transplant patients, a combination of cranberry juice and L-methionine reduces by more than 50% the incidence of UTI and also decreases the prevalence of symptomatic pyuric patients[35]. A Cochrane review of 10 good quality randomised controlled trials in over 1000 women suggests that cranberry juice decreases the number of symptomatic UTIs over a 12-mo period[36].

The evidence of efficacy is still less clear in children. Some studies have shown promising results in paediatric UTI prevention[37]. However, in a double-blind randomized placebo-controlled trial involving 255 children, while cranberry juice did not significantly reduce the number of children who experienced a recurrence of UTI, it was effective in reducing the actual number of recurrences and related antimicrobial use[38]. A recent Cochrane review showed that while cranberry juice decreases the number of symptomatic UTIs in women, there is still lack of such evidence in children[36]. It is also likely that its acidic nature reduces its palatability in children[36].

Glycosaminoglycans and sodium pentosanpolysulfate: The transitional epithelial cells at the surface of the urinary bladder secrete and bind to their surfaces one or more glycosaminoglycans that markedly reduce the ability of microorganisms to adhere to the mucosa, a prerequisite to cause a UTI[39]. Comparing the prevalence of UTI in intact mucin deficient rabbit bladders with those treated with sodium pentosanpolysulfate (PSP, a similar but synthetic substitute for the surface glycosaminoglycan), UTI were more frequent in mucin deficient bladders after exposure to bacteria. This suggests that the natural surface glycosaminoglycan(s) and the synthetic substitutes that reproduce their antiadherence effect appear to be protecting factors[40]. In human trials, the results have been conflicting. While no significant effect of sodium PSP was found compared to placebo in patients with interstitial cystitis and painful bladder disease[41], another study in patients with interstitial cystitis has shown a significant benefit from treatment with sodium PSP[42]. So far, no studies on its role in preventing UTI in children have been performed.

Bioflora modification

These alternatives are based on two mechanisms: Competitive exclusion and bacterial interference.

Competitive exclusion-lactobacillus and probiotics: The interest in studying probiotics for the prevention of UTI started after an animal study where the injection of five strains of periurethral uropathogenic organisms into the urinary bladder of female rats and then instilling an isolate of *Lactobacilli casei* GR1 from the urethra of a healthy woman resulted in decreased the development

of UTI by 84% up to 60 d later[43]. This experiment was underpinned by the concept of competitive exclusion as indigenous bacteria block the *in vitro* attachment of uropathogenic bacteria to human uroepithelial cells.

Women with recurrent UTI are believed to have pre-existing alterations in their normal vaginal microflora resulting in depletion of hydrogen peroxide (H_2O_2) containing Lactobacili which are protective against infections. Restoration of normal vaginal microflora through the use of (H_2O_2) containing lactobacillus probiotic has been investigated to test the hypothesis that it confers a protective effect against recurrent UTI in women. Intravaginal administration of Lactobacilli Crispatus suppository was established to be safe[44]. Vaginal suppositories of *Lactobacillus Crisptatus* reduced by 50% the incidence of UTI in 50 pre-menopausal women[45]. Oral probiotic yogurt, prepared from inoculating of *Lactobacillus acidophilus* (LA-5) and *Bifidobacterium* in heated milk, combined with antibiotic, was compared to antibiotic prophylaxis alone for prevention of UTI and it resulted in a significant reduction in the incidence of febrile UTIs in the third year of administration (although there was no difference in the first two years)[46]. Several studies challenged the benefit of *Lactobacillus*: it did not provide significant prevention of UTI In sick neonates in a neonatal intensive care unit[47]. And, when compared to *Vaccinium Macroponam, it* was not more effective in preventing UTI[48].

Bacterial interference: Animal studies showing the interference of *E. coli* with the growth of *Pseudomonas aeruginosa* in the bladder of male Wistar rats[49] raised the theory of bacterial interference as a therapeutic option. This was supported by the finding that asymptomatic bacteriuria, especially with *E. coli* 83972 (associated with symptom-free colonizations for long periods of time) protects against recurrent UTI. This observation has prompted clinical trials with deliberate colonization of the human urinary tract of patients with recurrent UTI which has resulted in a subjective benefit and in less UTI requiring treatment in colonized patients[50]. Similarly, UTI commonly occurs in patients with spinal cord injury as their bladder, particularly in the presence of an indwelling bladder catheter, can become colonized by a variety of organisms, including benign colonizing bacteria which are often left untreated because they may provide some protection against symptomatic infection with more pathogenic microbes. As a result, intentional colonisation of the neurogenic bladder in patients with spinal cord injury with a non-pathogenic strain of *E. coli* such as *E. coli 83972*[51-53] or *E. coli HU21117*[54] was attempted and was shown to reduce the risk of symptomatic UTI with pathogenic *E. coli* in these patients and was safe.

Vaccination

As deletion of the O antigen ligase gene, waaL, from the uropathogenic *E. coli* isolate NU14 results in a strain that stimulates urothelial cytokine secretion, NU14 DeltawaaL was tested as a vaccine for UTI in mice *via* instillation into the bladder as was shown to protect mice against challenge with a broad range of clinical uropathogenic *E. coli* isolates and produced immunity that lasted 8 wk. It is therefore a candidate live-attenuated vaccine for the treatment and prevention of acute and recurrent UTI by caused by uropathogenic *E. coli*[55,56]. Human trials have not been performed so far.

Voiding habits

Non-neurogenic neurogenic bladder, first described in 1986[57], is a disorder of functional bladder obstruction causing urinary retention and altered bladder anatomy that may lead to upper urinary tract dilatation and scarring[58]. Dysfunctional elimination syndromes are functional bowel and/or bladder disorders, including bladder instability, inability to effectively empty the bladder, infrequent voiding enuresis, UTI, incontinence, constipation or other voiding symptoms. They are common and often unrecognized in children with primary VUR[59]. Girls with recurrent UTI are more likely to have a high degree of dysfunctional elimination[60]. These syndromes are associated with delayed VUR resolution and an increased rate of breakthrough urinary tract infection, which may require ureteral reimplantation surgery[59]. These problems are not only important during childhood, but they may also have a negative impact on bladder and bowel function later life[61]. Objective assessment of symptoms severity, is required for screening and diagnosis purposes, confirmation of treatment results and follow up. It might also be useful for screening purposes[62].

Diagnosing and treating constipation as well as dysfunctional voiding are required to treat this condition[63]. Correcting constipation has been shown to decrease in the incidence of recurrent UTI[64]. In children with dysfunctional elimination, treating constipation with polyethylene glycol 3350 is successful, lacks significant side effects and is associated with good compliance and persistent constipation is associated with decreased resolution of voiding symptoms[65]. Biofeedback is an effective, non-invasive method of treating dysfunctional elimination syndrome with 80% success rate[66]. Children-directed biofeedback is also promising[67] and animated biofeedback, with pelvic floor muscle exercises, coordination of breathing and pelvic floor muscle contractions has been shown to be beneficial in improving dysfunctional elimination[68]. Sacral neuromodulation has been suggested for children with dysfunctional elimination syndrome whose symptoms are refractory to maximum medical therapy but should be cautiously used as it carries a significant risk of complications[69].

Ureteral re-implantation

The role of surgical treatment of VUR in the prevention

of UTI recurrences is well documented. Ureteral reimplantation in 205 infants (180 boys and 25 girls) with primary VUR reduced the frequency of febrile UTI reduced from 0.23538 before surgery to 0.00894 and 0.00081 per patient per month at six and 12 mo after surgery respectively, with no development of renal scarring on DMSA scan[70]. Several studies on a large number of children have shown absence of significant difference in renal growth between surgical ureteral re-implantation and medically treated children with primary VUR, both in previously scarred and in normal kidneys up to 10 years later, and, although pyelonephritis occurred significantly less often in surgically treated children, there was no significant difference in glomerular filtration rate nor in the development of hypertension[71-73]. A systematic meta-analysis was carried out by the Vesicoureteral Reflux Guideline Update Committee of the American Urological Association established to update the management of primary vesicoureteral reflux in children. A total of 2028 articles were reviewed, data were extracted from 131 articles including a total of 17972 patients. Guidelines for managing vesicoureteral reflux in children were issued but the lack of robust prospective randomized controlled trials limited the strength of these guidelines[74].

CONCLUSION

Several interventions, other than antibiotic prophylaxis, for the prevention of recurrent UTI have been tried and, although showing some promise, they do not provide so far a definitive effective answer.

Cranberry juice appears to be a promising and safe alternative with no serious adverse events. However its efficacy remains questionable in the pediatric population. Few studies are available on probiotics, but their efficacy is still debated for UTI prevention. Circumcision, a largely popular choice in certain countries, lacks good quality studies to prove its safety, and effectiveness. It was found to be particularly useful for children with low grade VUR and antenatal hydronephrosis, but the presence of many confounding factors requires further larger good quality studies to establish its efficacy. Glycosaminoglycan and sodium pentosanpolysulfate, found to be useful in animal models, have not been tested yet in humans. The benefit of surgical interventions, such as ureteral re-implantation, is confined to a particular group of patients, and the statistical significance of its efficacy remains questionable. Although improving voiding habits is certainly a beneficial approach, its effectiveness in isolation remains unproven. Vaccination is an attractive emerging option, but high quality large randomised controlled trials in humans are needed to look for its efficacy in UTI prevention.

Finding suitable alternatives to oral long-term antibiotic prophylaxis for UTI prevention still requires further high quality research of those seemingly promising interventions.

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EDITORIAL

Costimulatory blockade: A novel approach to the treatment of glomerular disease?

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Abstract

Costimulatory pathways (Cluster of differentiation 28, tumor necrosis factor-related, adhesion and T Cell Ig- and mucin-domain molecules) regulating the interactions between receptors on the T cells and their ligands expressed on several cell types, have a key role in controlling many immunological and non immunological processes. Indeed, accumulating evidence indicate that these molecules are involved in the pathogenesis of numerous conditions, such as allograft rejection, atherosclerosis, rheumatoid arthritis, psoriasis and renal diseases, including glomerulonephritis. Primary or secondary (*i.e.*, associated with infections, drugs or systemic diseases, such as systemic lupus erythematosus, diabetes, *etc*.) glomerulonephritis represent a group of heterogeneous diseases with different pathogenic mechanisms. Since costimulatory molecules, in particular CD80 and CD40, have been found to be expressed on podocytes in the course of different experimental and clinical glomerulonephritis, costimulation has been thought as a new therapeutic target for patients with glomerular diseases. However, although experimental data suggested that the blockade of costimulatory pathways is effective and safe in the prevention and treatment of glomerular diseases, clinical trials reported contrasting results. So, at this moment, there is not a strong evidence for the general use of costimulatory blockade as an alternative treatment strategy in patients with primary or secondary glomerulonephritis. Here, we critically discuss the current data and the main issues regarding the development of this innovative therapeutic approach.

Key words: Costimulation; Glomerulonephritis; Cluster of differentiation 80; Cytotoxic T-lymphocyte-associated antigen-4; Lupus nephritis; Abatacept; Proteinuria; Podocytes

Core tip: Glomerulonephritis refer to a group of renal disorders, primary or secondary to infections, drugs or systemic diseases, characterized by inflammation within the glomerulus. Among glomerular diseases there is a great clinical, histological and prognostic heterogeneity

and several different pathogenetic mechanisms have been implied. Current standard treatments include steroids and cytotoxic agents, which present important side effects and an unsatisfactory remission rate. Therefore, experimental and clinical research is addressed to the development of alternative therapies. Here, we critically discuss new therapeutic opportunities provided by the use of agents acting on the modulation of costimulatory pathways.

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COSTIMULATORY PATHWAYS

Costimulatory pathways regulating the interactions between receptors on the T cells and their ligands expressed on several cell types (including immunocompetent cells, fibroblasts, endothelial cells, *etc.*) play a crucial role in the modulation of immunological and non-immunological processes[1].

In particular, costimulation is essential for the full activation of naïve T cells after antigen-specific recognition, and without costimulation the T cell-antigen interaction results in anergy[2].

Different costimulatory families [Cluster of differentiation 28 (CD28), tumor necrosis factor (TNF)-related, adhesion and T Cell Ig- and mucin-domain (TIMs) molecules], characterized by structural and functional analogies, have been described. These molecules can interact with each other either up- or down-regulating T cell activation[3] (Table 1).

Among the identified costimulatory molecules, the best characterized are the CD28:B7 and the TNF-related families. The CD28:B7 family includes the following receptor-ligand pairs: CD28/CTLA4:CD80/CD86, induced costimulatory molecules (ICOS:ICOSL) and the programmed death-1 pathway (PD-1:PD-L1/PD-L2)[4]. CD28 is a disulfide-bound molecule that belongs to the immunoglobulin superfamily and is constitutively expressed on T cells[5].

Its interaction with CD80 (B7.1) and CD86 (B7.2), expressed on the surface of antigen-presenting cells (APCs), leads to the full activation of T cells[6]. Conversely cytotoxic T-lymphocyte-associated antigen-4 (CTLA4), a structural homologous of CD28 with a higher avidity for CD80 and CD86, acts as a negative regulator of T cells[7].

TNF superfamily comprises: CD40:CD40L, OX40:OX40L, CD30:CD30L, CD27:CD70, CD137:CD137L, *etc*. CD40 is mainly expressed on B-cells, but also on monocytes, dendritic cells, endothelial cells, smooth muscle cells and fibroblasts[8]. The engagement of CD40 with its ligand, CD40L (CD154), leads to B cell expansion and differentiation and it is decisive in the regulation of APCs and dendritic cells functions[9]. It is important to underline that costimulatory molecules, expressed by a broad variety of cells, seem to be involved in the pathogenesis of numerous conditions, such as atherosclerosis, rheumatoid arthritis, psoriasis and renal diseases, including allograft rejection and glomerulonephritis[10-14].

The insights regarding the contribution the costimulatory molecules in these conditions has not only allowed elucidating important regulatory mechanisms, but has also provided novel targets for therapeutic interventions[15].

Table 1 Immunomodulatory effects of costimulation pathways

Family	Ligand	Receptor	Effects on immune cells
CD28	CD80 (B7.1)/	CD28	+
	CD86 (B7.2)	CTLA-4	-
	ICOSL	ICOS	+
	PDL1	PD-1	-
TNF-related	CD40	CD40L (CD154)	+
	OX-40	OX-40L	+
Adhesion molecules	ICAM-1	LFA-1	+
TIM	TIM4/9	TIM1/3	+/-

Costimulatory pathways may influence immune response through stimulatory (+) or inhibitory (-) signals. Ligands may be present on antigen-presenting cells, including B-lymphocytes and dendritic cells, but also on muscle, endothelial, fibroblast, platelets and epithelial-derived cells. Receptors are mainly expressed on T-cells[48]. CTLA-4: Cytotoxic T-lymphocyte-associated antigen-4; ICOS: Induced costimulatory molecule; PD-1: Theprogrammeddeath-1; LFA-1: Lymphocyte function-associated antigen 1; ICAM-1: Intracellular adhesion molecule 1; TIM: T cell Ig and mucin.

COSTIMULATION AND GLOMERULONEPHRITIS

Glomerulonephritis refer to a group of renal disorders, primary or secondary to infections (human immunodeficiency virus, hepatitis C virus, *etc.*), drugs and systemic diseases (for example, systemic lupus erithematosus-SLE, cancer and diabetes), characterized by inflammation within the glomerulus[16].

Among glomerular diseases there is a great clinical, histological and prognostic heterogeneity and several different pathogenic mechanisms are implied, including podocyte damage, immunoglobulin deposition and immune cell infiltration[17]. During the last years growing evidence suggest a role for costimulatory molecules also in this specific setting.

In particular, CD80 expression has been detected in podocytes, which integrity is essential to maintain a regular glomerular function[18].

Indeed, in experimental models of genetic, drug-induced, immune-mediated and bacterial toxin-induced kidney diseases, CD80 overexpression on podocytes might be harmful for glomerular permeability, disturbing the slit diaphragm and down-regulating podocytes-β1

integrin activation, finally leading to the development of proteinuria and loss of renal function[19,20]. The crucial role of CD28:CD80 pathway in the pathogenesis of glomerular diseases is also confirmed by the evidence that CD80 knockout mice present an attenuated form of proliferative glomerulonephritis, associated with a significant reduction of renal tissue lesions[21].

Moreover, the use of monoclonal antibodies targeting CD28 or CTLA-4 was effective in treating and preventing different forms of experimental nephritis, including lupus-like nephritis[22]. Interestingly, similar results were also found in human glomerulonephritis. In particular, a significant increase in CD80 podocyte expression and urinary excretion has been reported in patients with minimal change disease (MCD) in relapse compared to those in remission or with focal segmental glomerulosclerosis (FSGS)[23,24]. Similarly, patients with proliferative lupus nephritis present a strong podocyte surface expression of CD80[20].

Beyond CD28:CD80 pathway, also costimulatory molecules of TNF-related family, *i.e.,* CD40:CD154, have been found expressed in renal tissue in the course of both experimental and human glomerular diseases. CD40 was isolated in murine models of proteinuric disease, such as membranous glomerulonephritis, lupus nephritis and necrotizing nephritis[25]. Moreover, glomerular and tubular CD40 expression was up-regulated in human lupus nephritis and in other inflammatory renal diseases, being associated with the presence of CD40L+ mononuclear cells[26]. Furthermore, the inhibition of CD40 pathway through the administration of a CD40-Ig fusion protein or anti-CD40L antibodies prevented the development of proteinuric kidney diseases in mice[27,28].

COSTIMULATORY BLOCKADE AS A NOVEL TREATMENT FOR GLOMERULONEPHRITIS

As a consequence of the role of costimulation in the pathogenesis of several pathological conditions, costimulatory blockade has been thought as a new rational therapeutic approach[29]. Therefore different strategies, mainly based on the design of specific monoclonal antibodies (mAbs) interfering with these critical pathways, have been tested. However, the clinical development of the majority of these new strategies is currently suspended for safety concerns.

This is, for example, the case of anti-CD40L mAb, which although effective in the prevention of glomerular diseases and renal allograft rejection in murine and primate experimental models, significantly increased the occurrence of thromboembolic events[27,30-32]. More severe complications occurred during the development of anti-CD28 mAbs. Indeed, six healthy volunteers enrolled in a phase I clinical trial and treated with a humanized superagonistic anti-CD28 mAb, developed a life-threatening systemic inflammation due to massive cytokine release, determining the complete abandon of this approach[33]. A more promising strategy- the only one that has found clinical applications so far- seems to be the development of CTLA-4 immunoglobulin fusion proteins. These proteins are composed by an extracellular portion of human CTLA4 plus a Fc part of human IgG1, which, binding CD80 and C86 with high avidity, prevent CD28 ligation, acting as potent inhibitors of CD28:CD80/CD86 pathways[34,35]. Abatacept, which has been approved by FDA for the treatment of rheumatoid arthritis in 2005, and its derivate, Belatacept, belong to this category of drugs.

Belatacept has been extensively studied mainly in the experimental and clinical setting of renal transplantation.

Belatacept was evaluated in 2 open-label, randomized, multicenter, controlled phase 3 studies: the Belatacept Evaluation of Nephroprotection and Efficacy as First-line Immunosuppression Trial (BENEFIT) and BENEFIT-EXT ("extended criteria").

These studies showed that Belatacept was non-inferior to Cyclosporine in terms of patient and graft survival, being associated to a better graft function and a reduced incidence of chronic nephropathy[36,37]. Hence, although the administration of belatacept was not exempt from adverse effects, in 2011 it was approved by Food and Drug Administration (FDA) as the first costimulatory blocker for use in renal transplantation[38].

Regarding the specific setting of primary and secondary glomerulonephritis, instead, only Abatacept has been used in clinical studies with discordant results.

Two recent randomized trials investigated the safety and efficacy of Abatacept in addition to standard treatments in patients with lupus nephritis.

A twelve months blind multicentre trial, performed by Furie *et al*[39], enrolled 298 patients with active lupus nephritis and proteinuria, randomized to receive corticosteroids and Mycophenolate mofetil in association with Abatacept (30 mg/kg loading for 3 mo, followed by 10 mg/kg), Abatacept (10 mg/kg) or placebo. The authors found that the treatment with Abatacept was associated with a reduction of antiDNA antibody, C3 and C4 levels and proteinuria. However, there were not significant differences in the time to reach a complete response and in the proportion of subjects with confirmed complete response after 52 wk of follow-up among the three groups.

Similar results have been reported by Askanase *et al*[40] who evaluated the efficacy of Abatacept *vs* placebo added to a standard treatment regimen with Cyclophosphamide followed by Azathioprine in 134 patients with active lupus nephritis. They also found no significant differences between the groups in terms of number of patients reaching and/or maintaining complete or partial response.

So, even if previous studies reporting the strong expression of podocyte CD80 in human proliferative lupus nephritis appeared promising, the results of these clinical trials have unexpectedly called into question the

utility of Abatacept in patients with SLE.

Abatacept has been also studied in patients with primary glomerulonephritis.

In a recent paper, Yu *et al*[41] tested Abatacept in 5 patients with FSGS (4 with recurrent FSGS after kidney transplantation and 1 with primary FSGS) who presented positive CD80 (B7.1) immunostaining of podocytes in kidney-biopsy specimens.

After treatment with Abatacept all these patients presented a partial or complete remission, expressed as a significant reduction of serum creatinine and/or proteinuria. Interestingly, the authors provided also a rationale for the beneficial effects of Abatacept, demonstrating that the drug *in vitro* blocks podocyte migration and stabilizes β1-integrin activation in podocytes[41].

Although exciting, these results have been criticized for several important methodological issues[42,43]. First of all, it should be considered that the 4 patients with recurrent FSGS underwent intensive plasmapheresis, aimed to remove putative circulating permeability factors. Thus, it is not possible to recognize if the disease remission was due to this treatment independently of the use of Abatacept. Moreover, subsequent reports arose doubt about the immunostaining techniques used to detect CD80 in renal tissue, highlighting the lack of any negative controls. In particular, Larsen *et al*[44] tested the presence of CD80 in 60 renal biopsy specimens from patients with different proteinuric glomerular diseases with two immunostaining methods (immunoperoxidase and immunofluorescence). The authors found that for both staining techniques and in all cases, CD80 was undetectable within podocytes. The presence of so contrasting results among experimental and clinical trials raises doubt about the potential role of Abatacept in patients with proteinuric glomerulonephritis[45].

To be thorough, it has to point out that the efficacy of Abatacept in the treatment of MCD has been recently reported in a single case[46].

Considering the overall above reported data, we might infer that, although the podocyte CD80 pathway seems to have an important role in some proteinuric glomerular diseases, clinical results suggest that current therapeutic strategies do not warrant a satisfactory control of glomerulonephritis.

CONCLUSION

The critical analysis of the currently available data suggests some conclusions: (1) costimulatory pathways might be implied in the pathogenesis of glomerulonephritis, especially the forms associated with proteinuria and nephrotic syndrome; (2) the development of drugs targeted to block costimulation is of great potential utility, also considering that the current available therapeutic options are limited[47]; (3) clinical trials have shown insufficient or, at least, contrasting effects of this kind of approach in the achievement of therapeutic targets and disease remission.

So, it appears clear that further molecular, cellular and clinical studies, including the design and evaluation of new drugs and exploration of new pathways, should be performed before considering costimulatory blockade as a valid alternative treatment in the general population of patients with glomerulonephritis.

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EDITORIAL

Clinical neurological examination *vs* electrophysiological studies: Reflections from experiences in occupational medicine

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Abstract

Seventy-five percent of upper limb disorders that are related to work are regarded as diagnostically unclassifiable and therefore challenging to the clinician. Therefore it has been generally less successfully to prevent and treat these common and frequently disabling disorders. To reach a diagnosis requires the identification of the responsible pathology and the involved tissues and structures. Consequently, improved diagnostic approaches are needed. This editorial discusses the potentials of using the clinical neurologic examination in patients with upper limb complaints related to work. It is argued that a simple but systematic physical approach permits the examiner to frequently identify patterns of neurological findings that suggest nerve afflictions and their locations, and that electrophysiological studies are less likely to identify pathology. A diagnostic algorithm for the physical assessment is provided to assist the clinician. Failure to include representative neurological items in the physical examination may result in patients being misinterpreted, misdiagnosed and mistreated.

Key words: Neurological examination; Electrophysiology; Work-related disorders; Occupational medicine; Nerve afflictions; Upper limb disorders

Core tip: Patients with work-related upper limb disorders should be subjected to a systematic upper limb examination including neurological items with the main focus on muscle strength testing. A refined version of the classical neurological upper limb examination can be rewarding because it permits the clinician to frequently identify patterns in accordance with nerve afflictions with specific locations. This examination is suitable in any clinical setting because it is simple, inexpensive, noninvasive, and highly reproducible.

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An estimated three quarters of upper limb disorders that are related to work are regarded as diagnostically unclassifiable[1] and consequently these conditions remain a diagnostic challenge to the physician. Therefore evidence-based prevention and treatment have been largely unsuccessful. To reach a diagnosis requires the identification of the tissues and specific structures that are involved, and also of the pathology that causes the condition. To achieve this goal, improved diagnostic approaches to these frequent and disabling conditions should be applied.

Frequently, patients with work-related upper limb disorders have pain with characteristics that suggest that the pain is neuropathic. The pain may be accompanied by sensory abnormalities such as paraesthesia, subjective weakness or heaviness and/or tactile dysfunction. The combination of a neuropathic pain with motor and sensory symptoms suggests that the nervous system is involved and therefore that the upper limb peripheral nerves should be included in the physical examination. Still, clinicians and also researchers tend to ignore this possibility and to rather attribute these conditions to pathology located in muscles, tendons or insertions. Even if involvement of peripheral nerve(s) is considered, the attention tends to be directed - and limited - to carpal tunnel syndrome and afflictions of a cervical root. The intermediate portion of nerve of a length approaching one meter, and other potential locations of focal neuropathies receive less attention.

Focal neuropathies cause rather specific neurological patterns: If muscles are innervated distally to a nerve-lesion they are likely to be weak; the sensibility in supplied cutaneous territories will be altered; there will be abnormal soreness where the nerve trunk is affected. The classical neurological examination is based on these principles and all physicians have been trained in its execution. Still, there seems to be reluctance to perform a detailed neurological examination in upper limb patients with work-related complaints. This is particularly the case with respect to the portions of the upper limb nerves that are located proximally such as in the forearm, arm and shoulder. To the experience of the author this inadequacy applies to physicians in any specialty including neurology. Performing a detailed clinical neurological examination and to interpret its outcome may be regarded as difficult, and this examination may also be assumed to be time consuming. It is true that relevant benefit of the examination requires knowledge of anatomy such as the location of nerves and their innervation patterns. This has been previously learned but might be forgotten. To achieve a neurological diagnosis such as the identification of a focal nerve affliction of the upper limb, the neurological examination may also be perceived as less rewarding than paraclinical studies. Although this view is not justified physical neurological upper limb examinations are rarely more than basic. Rather than performing a detailed examination it may be easier for the clinician to refer the patient to electrophysiological examination (and frequently to MR-imaging - in particular of the cervical spine) and so to leave the diagnostic work to others.

This choice may be justified if electrophysiological studies of the upper limb nerves reflect the truth better than the traditional neurological examination. Clinicians - including many neurologists - tend to view electrophysiological (and imaging) studies as superior to their own physical examination and judgement. Consequently, the outcome of a physical examination based on patterns of neurological abnormalities suggesting focal peripheral neuropathy is likely to be regarded as less valid if electrophysiological studies do not identify abnormalities. However, according to the scientific literature, the superiority of electrophysiology has not been demonstrated and certainly not for disorders of the upper limb studied in an occupational context. While electrophysiological studies tend to be generally viewed as "golden standard" for peripheral neuropathy their sensitivity - in particular with minor nerve afflictions such as may be the case with work-related upper limb complaints - is limited because nerve lesions may be mixed and partial with few myelinated fibres intact and reinnervation taking place. Therefore the electrophysiological findings may be entirely normal[2]. The potentials of a refined electrophysiological assessment are acknowledged but the application of expanded techniques is also very time consuming and consequently expensive, and therefore rarely applied. There is also agreement that a detailed neurological physical examination should precede an electrophysiological study of the peripheral nerves and guide its content. Evidently, it has no sense to study electrophysiologically the median nerve in the carpal tunnel when a nerve affliction is located elsewhere. Some common locations of upper limb nerve entrapment can only rarely be identified by electrophysiological studies, *e.g.*, radial tunnel syndrome[3], pronator syndrome[4] and brachial plexopathy[5].

It is essential that the physical examination focuses on all nerves of relevance and on the entire length of nerves from the roots to their muscular and cutaneous supply. That means that the examiner should include neurological items that are representative to nerve afflictions with any location that one would expect. The physical neurological examination should be reliable and also valid, meaning that it should be capable to identify abnormalities in symptomatic limbs and exclude abnormalities in healthy limbs, respectively.

A semiquantitative detailed upper limb neurological examination, which has been developed for this

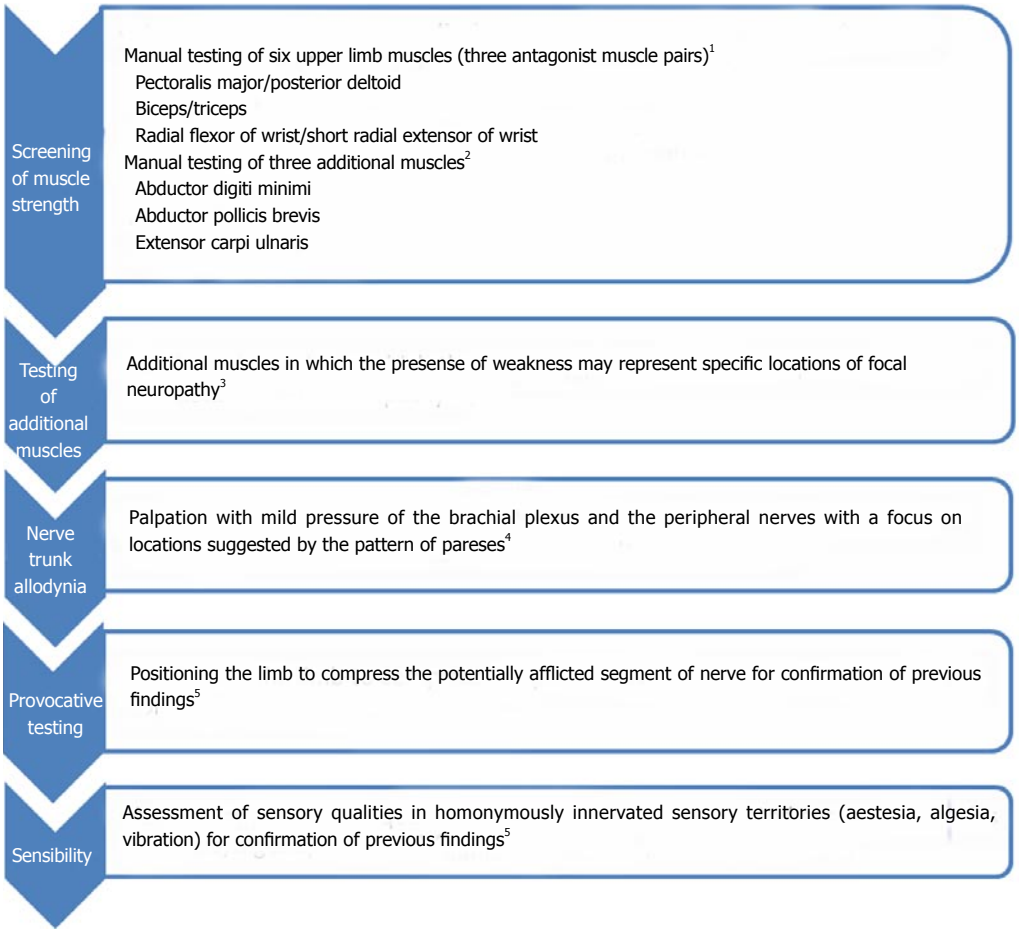

Figure 1 An algorithm for an upper limb neurological examination in patients with work-related upper-limb complaints. [1]Manual testing of six upper limb muscles is a sensitive measure, which can suggest the presence of the majority of upper limb nerve afflictions but confirmation of focal neuropathy requires the demonstration of mechanical nerve trunk allodynia at the location(s) that are suggested by the pattern of pareses[11]. The combination of manual muscle testing and nerve trunk palpation is able to increase the specificity of the neurological examination; [2]Screening of strength in the six muscles applied for the initial screening cannot identify focal neuropathy at three common locations: Ulnar neuropathy will result in weakness in the abductor digiti minimi, carpal tunnel syndrome will be associated with paretic abductor pollicis brevis muscle, and radial tunnel syndrome causes weakness in the extensor carpi ulnaris muscle. Therefore manual testing of these three muscles is also recommended. If all nine muscles are of normal strength, a peripheral nerve affliction is unlikely. If weaknesses are found, additional muscles should be tested[12]; [3]The muscles selected to be recommended for screening are those that according to the author are the more rewarding in the diagnosis of upper limb nerve afflictions. Depending on the clinical situation nerve afflictions of less frequency may be looked for by examining additional muscles[7]; [4]The identification of mechanical allodynia at location(s) suggested by the pattern of pareses will improve the specificity of the examination and contribute to its validity[11]; [5]Provocative testing and assessment of sensory qualities serve to reassure the examiner of the neurological findings. Examples of the former are the well-known Phalen sign with carpal tunnel syndrome and the hyperabduction test with an infraclavicular brachial plexus affliction. Passive compression of the posterior interosseous nerve by passive forearm hyperpronation will provoke the pain associated with a radial tunnel syndrome. While upper limb tension tests have been developed to indicate bias towards the median, radial, and ulnar nerves, respectively[13], they cannot, however, determine the level(s) of afflicted location(s) along these nerves. The same is the case for sensory assessment. Therefore manual muscle testing is the key to the neurological upper limb assessment.

purpose consists of manual strength-testing in selected individual and representative muscles[6]. It also contains of an assessment of sensory deviations from normal in representative territories, which are homonymously innervated. Finally, the presence of allodynia of nerve trunks with mild palpation should be studied at the location(s) where nerves may be affected[7]. This low-tech examination is rapid to perform and requires no equipment beside a needle and a 256 Hz tuning fork.

When this examination was carried out on patients that were referred for assessment in a hospital department of occupational medicine there was a high occurrence of patterns of neurological findings. These patterns were in accordance with the topography of the nerves and their muscular and sensory innervation – and they were also frequently demonstrated in upper limb patients that could not be diagnosed by conventional means. The construct validity of this approach in terms of interrelations of nerve afflictions with various location has previously been demonstrated[8]. There was a high inter-rater reliability of the identification of neurological patterns[7]. The validity of this approach was further indicated by demonstrating that the presence of neurological patterns was related to the presence of symptoms[9]. The most frequent location of nerve afflictions in the upper limb was at the infraclavicular brachial plexus (behind the minor pectoral muscle below the clavicle). This location of nerve affliction was often

found in combination with median neuropathy (just proximally to and medially to the elbow joint) and radial (posterior interosseous) neuropathy (at the Arcade of Frohse) at elbow level. Whether diagnosed by criteria that included a thorough neurological examination or by conventional diagnostic criteria, neuropathic upper limb conditions could also be identified as the most frequent among patients in general practice[10].

It is frequently frustrating to experience when a patient with peripheral upper limb nerve affliction(s) with certain location(s) diagnosed by this examination is subsequently examined by a neurologist. After a mostly limited neurological examination and an electrophysiological study targeting a few selected parameters, the neurologist is likely to conclude the absence of a neurological condition and to interpret the patient's complaints and findings (*e.g.*, "pain induced weakness" or "sensory deviation with a non-dermatomal extension") as either due to a disorder located to muscles or to be functional and without any somatic origin. These patients are likely to be misinterpreted, misdiagnosed and mistreated. Furthermore, the legal authorities seem to trust the basic examination by the neurologist rather than to appreciate a detailed neurological examination.

I regard the assessment of individual muscle strength as the most important part of the clinical neurological tests. I would therefore suggest clinicians who see upper limb patients to routinely integrate in their physical examination a screening approach consisting of manual muscle testing of six to nine representative upper limb muscles (Figure 1). This approach is sensitive and permits the identification of patients that should be physically examined further to determine the location of an upper limb nerve affliction[11]. Any physician can easily learn to manually assess the strength in individual muscles[6,11]. This part of the examination is therefore feasible in any clinical setting whether it be in industrialized countries or in the developing world.

A correct diagnosis is essential for targeted preventive intervention at workplaces as well as for treatment, which may follow the concepts of neuromobilisation[13-15]. There is increasing evidence of an effect of nerve mobilisation in the treatment of upper limb nerve afflictions[14]. Neurolytic surgery for upper limb nerve afflictions has been undertaken for years but its success depends of a precise location of the affliction.

Including a systematic neurologic examination in the diagnostic physical approach to patients with work-related upper limb disorders may eventually constitute a step towards improved prevention but this remains to be demonstrated. The first step would be to demonstrate risk factors in work as has been done for certain neuropathic upper limb conditions such as, *e.g.*, radial tunnel syndrome[16] and brachial plexopathy[17]. Next would be to see whether the removal or reduction of these risk factors would reduce the occurrence.

I would caution against blind faith in diagnostic tools such as electrophysiological studies. As demonstrated by others, it is not automatically the best or safest choice to trust device outputs that have a potential for flawed measurement. Trained judgement should be applied when interpreting results generated from devises[18] such as results from electrophysiological studies. The clinician should know their potentials and limitations, and be able to assess whether they are better or inferior than the clinical examination.

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EDITORIAL

Cross-reactivity between aeroallergens and food allergens

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Abstract

In patients with respiratory allergy, cross-reactivity between aeroallergens and foods may induce food allergy, symptoms ranging from oral allergy syndrome to severe anaphylaxis. Clinical entities due to IgE sensitization to cross-reactive aeroallergen and food allergen components are described for many sources of plant origin (pollen-food syndromes and associations, such as birch-apple, cypress-peach and celery-mugwort-spice syndromes, and mugwort-peach, mugwort-chamomile, mugwort-mustard, ragweed-melon-banana, goosefoot-melon associations), fungal origin (*Alternaria*-spinach syndrome), and invertebrate, mammalian or avian origin (mite-shrimp, cat-pork, and bird-egg syndromes). Clinical cases of allergic reactions to ingestion of food products containing pollen grains of specific plants, in patients with respiratory allergy to *Asteraceae* pollen, especially mugwort and ragweed, are also mentioned, for honey, royal jelly and bee polen dietary supplements, along with allergic reactions to foods contaminated with mites or fungi in patients with respiratory allergy to these aeroallergens. Medical history and diagnosis approach may be guided by the knowledge about the diverse cross-reacting allergens involved, and by the understanding of these clinical entities which may vary significantly or may be overlapping. The association between primary IgE sensitization with respiratory symptoms to inhaled allergens and food allergy due to cross-reactive allergen components is important to assess in allergy practice. The use of molecular-based diagnosis improves the understanding of clinically relevant IgE sensitization to cross-reactive allergen components from aeroallergen sources and foods.

Key words: Cross-reactivity syndromes and associations; Aeroallergens; Food allergens

Core tip: Many different syndromes and associations due to cross-reactivity between aeroallergens and food allergens of plant, fungal and animal origin have been described. Significant examples are pollen-food syndromes or associations, along with mite-shrimp, cat-pork, and bird-egg syndromes, but rare or more complex clinical entities must also be discussed. It is important to underline the impact of relevant cross-reactivities between aeroallergens and food allergens and of molecular-based allergy diagnosis in clinical practice.

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INTRODUCTION

Respiratory allergies affect 10%-30% of adults and children worldwide[1,2], while food allergy is estimated to affect more than 1%-2% and less than 10% of the population[3]. Allergic rhinitis, asthma and food allergies have significant detrimental effects on health-related quality of life, family economics, social interactions, school and work attendance[4-7]. There is a high co-occurrence of food allergy with other atopic diseases, including allergic rhinitis and asthma. Evidence of respiratory allergy may indicate an increased risk of IgE-mediated food allergy. Moreover, underlying asthma, regardless of severity, has been associated with increased risk of severe reactions and even death caused by food allergy[3].

This editorial underlines the importance of the IgE sensitization *via* the respiratory route to aeroallergens and food allergy due to cross-reactivities between some allergen components. This phenomenom should be distinguished from the common food allergy without sensitization to cross-reactive aeroallergens, in which heat- and enzyme-resistant class 1 food allergens induce allergic sensitization *via* the digestive tract, typically being responsible for systemic allergic reactions. Class 2 food allergens are more heat-labile and susceptible to digestion and therefore do not cause gastrointestinal sensitization, but instead provoke allergic reactions in already sensitized patients to cross-reactive aeroallergens through the respiratory route. Typically, pollen-food syndromes are produced by class 2 food allergens. In contrast to class 1 food allergy which mainly affects young children, class 2 food allergy is observed especially in adults as a consequence of sensitization to cross-reactive aeroallergens[8-10]. This traditional classification has a more modern changed approach from a molecular allergy point of view. Important allergen components families involved in cross-reactivity between aeroallergens and food allergens are presented in Table 1. The clinical expression for IgE sensitization to PR-10 proteins and profilins is mainly oral allergy syndrome.

Cross-reactivity is an immune-mediated phenomenon of an IgE antibody recognizing, binding, and inducing an immune response to similar allergenic molecules (homologues). IgE cross-reactivity often occurs between allergenic molecules in closely related species or well preserved molecules with similar function present in widely different species, belonging to the same protein family[3,11,12].

The clinical relevance of cross-reactivity seems to be influenced by a number of factors including the host immune response against the allergen, exposure and the allergen itself[13]. Cross-reactivity is important for various reasons, such as its immunologic basis, particularly in relation to the regulation of allergic sensitization, the risk of allergic cross-reactivity to novel foods and the identification of the patterns of cross-reactivity, because they often, but not always, may reflect the pattern of clinical sensitivities. Cross-reactivities involve clustering cross-reactive allergens or family-restricted homologous molecules, panallergens and cross-reactive carbohydrate determinants (CCDs)[14]. Panallergens are cross-reactive allergens, belonging to protein families well preserved throughout many widely different species, able to trigger IgE antibody binding[12]. Panallergens are ubiquitous proteins responsible for IgE cross-reactivity to a wide variety of related and unrelated allergenic sources. IgE cross-reactivity is usually approached from an allergen-perspective, meaning that cross-reactivity is a consequence of structural similarity between homologous proteins, which is translated into conserved sequence regions, three-dimensional folding and function[15]. CCDs are carbohydrate moieties of glycoproteins that induce the production of highly cross-reactive IgE. Many plant and invertebrate allergens are glycoproteins containing carbohydrate moieties called N-glycans that interfere with *in vitro* specific IgE determinations. Anti-CCD IgE biomarker indicate the presence in serum of IgE directed against carbohydrate epitopes. Grass pollen sensitization is the most common cause of CCDs sensitization in food allergic patients, anti-CCD IgE antibodies are highly cross-reactive with CCD monovalent peanut allergens, but does not induce clinical symptoms. CCDs rarely cause allergic reactions, but are an important cause of cross-reactivity for *in vitro* specific IgE assays for CCD-containing allergens from pollen, plant foods, insects and venoms. The use of CCD-free recombinant allergen components may be of utility in such cases[16-20].

In general, the term cross-reactivity should be used to describe defined clinical features revealing the reactivity to a source without previous exposure[13,21]. The comprehensive term co-recognition, including by definition cross-reactivity, could be usefully adopted to define the large majority of the IgE reactivity where co-exposure to a number of sources bearing homologous molecules does not allow the identification of the sensitizer. The CCD-IgE co-recognition of similar carbohydrate structures on unrelated sources may lead to *in vitro* false positive results in diagnostic tests[13]. Despite having high sequence homology in some cases, the ability of cross-reactive allergens to mediate clinical allergic reactions is highly variable, and often depends on the specific allergen sources involved. In addition, cross-reactivity between allergens may cause covariation of sensitization, such as a higher frequency of sensitization to two or more allergens than the expected frequency[3,22]. Immunologically, cross-reactivity is distinct from co-sensitization in which

Table 1 Important allergen components families involved in cross-reactivity between aeroallergens and food allergens[9,11,12,20]

Allergen components families (sensitivity to heat and proteases)	Examples of relevant allergen components involved (allergen sources)
PR-10 proteins, Bet v 1 homologues	Bet v 1, Aln g 1 (tree pollen)
(sensitive to heat and digestion)	Mal d 1, Pru p 1, Api g 1, Gly m4 (fruits, vegetables, legumes)
Profilins, Bet v 2 homologues	Bet v 2, Ole e 2 (tree pollen), Che a 2, Art v 4, Amb a 8 (weed pollen)
(sensitive to heat and digestion)	Api g 4, Dau c 4, Pru p 4, Cuc m 2, Mus xp 1, Sin a 4 (vegetables, fruits, seeds)
Lipid transfer proteins	Pla a 3, Ole e 7, Art v 3, Amb a 6 (tree and weed pollen)
(stable to heat and digestion)	Api g 2, Pru p 3, Cuc m LTP, Mus a 3, Sin a 3 (vegetables, fruits, seeds)
Tropomyosins	Der p 10, Bla g 7 (house dust mites, insects)
(stable to heat and digestion)	Pen m 1, Myt e 1 (crustaceans, mollusks)
Serum albumins	Fel d 2, Can f 3, Equ c 3 (cat, dog and horse serum albumins)
(fairly sensitive to heat and digestion)	Bos d 6, Sus s 6 (bovine and porcine serum albumins)

genuine sensitization to more than one allergen sources is not due to cross-reactivity, being not mediated by shared epitope-specific antibodies[3,12].

Accurate epidemiologic data on the prevalence of clinical cross-reactivities between aeroallergens and food allergens are generally limited by the lack of large, controlled population-based studies, incorporating oral food challenges. In adults, up to 80% of all cases of food allergy are preceded by sensitisation (clinical or subclinical) to aeroallergens, food allergic symptoms being caused in these patients by cross-reactions between ingested food and inhaled allergens[23]. Even in children, it is suggested that cross-sensitization may be found in up to 25% of cases[24].

CROSS-REACTIVITY BETWEEN AEROALLERGENS OF PLANT ORIGIN AND FOOD ALLERGENS

Pollen-food syndromes and associations are food allergies affecting pollen-sensitized individuals, that have become the most prevalent types of food allergy in European adolescents and adults, affecting about 5% of the population in central Europe. In United Kingdom, pollen-food syndrome overall prevalence is about 2%, in South-Eastern England urban practice being slightly over 4%. The symptoms of pollen-food allergy syndromes range from oral allergy syndrome to severe anaphylaxis, and the foods involved are of vegetal origin, mostly fruits and vegetables, eaten raw[9,25,26]. Pollinosis patients often display adverse reactions upon ingestion of plant-derived foods as a result of IgE cross-reactive epitopes shared by pollen and food allergen sources[1].

The role of an allergy specialist in recognizing and assessment of pollen-food syndromes and associations is essential. Many purified native and recombinant allergen components have been obtained in order to use them for a detailed molecular component-resolved diagnosis of the genuine sensitization and cross-reactivities profiles, and for a more accurate prescription of allergy immunotherapy[20,27,28].

Specific IgE antibodies to recombinant and native specific allergen components from trees, grasses and weeds pollen are important to differentiate the true sensitization profile in patients with multiple pollen sensitizations, as described below[20,27-32].

Tree pollen-specific allergen components are mentioned for anemophilous trees/shrubs belonging to the *Betulaceae* family: rBet v 1, a 17 kDa pathogenesis-related protein PR-10 with ribonuclease activity from the pollen of birch *Betula verrucosa*, cross-reactive with other *Betulaceae* pollen PR-10 components with about 70% identity to it (alder *Alnus glutinosa* rAln g 1, hazel *Corylus avellana* rCor a 1); *Oleaceae* family: nOle e 1 and rOle e 1, a 19-20 kDa trypsin inhibitor from the pollen of olive *Olea europaea*; *Platanaceae* family: rPla a 1, a 18 kDa invertase inhibitor, and nPla a 2, a 43 kDa polygalacturonase, from the pollen of plane tree *Platanus acerifolia*; *Cupressaceae* family: nCup a 1, a 43 kDa pectate lyase, from the pollen of cypress *Cupressus arizonica*, cross-reactive with other *Cupressaceae* pollen pectate lyase components (cedar *Criptomeria japonica* nCry j).

Grass pollen-specific rPhl p 1 (27 kDa beta-expansin), rPhl p 5b (32 kDa ribonuclease), and natural timothy grass (*Phleum pratense*) extract are used to identify grass pollen allergy. Specific IgE against rPhl p 1 is a *Poaceae* family-specific biomarker for genuine sensitization to grass pollen, and against rPhl p 2, rPhl p5 and rPhl p 6 are *Pooideae* subfamily-specific biomarkers for true sensitization to temperate grass pollen. Specific IgE antibodies to nCyn d 1 (beta-expansin of Bermuda grass *Cynodon dactylon)*, a warm climate grass-specific native pollen allergen component, represent biomarkers of genuine sensitization to *Chloridoideae* subfamily grass pollen.

Weed pollen-specific allergen components are described for herbaceous plants belonging to the *Asteraceae* (*Compositae*) family: nArt v 1, a 28 kDa defensin from the pollen of mugwort *Artemisia vulgaris* and nAmb a 1, a 38 kDa pectate lyase from the pollen of short ragweed *Ambrosia artemisiifolia* var. *elatior*; *Plantaginaceae* family: rPla l 1, a 17 kDa Ole e 1-like trypsin inhibitor from the pollen of plantain *Plantago lanceolata*; *Urticaceae* family: rPar j 2, a 14 kDa lipid transfer protein (LTP) from the pollen of wall pellitory *Parietaria*

Table 2 Significant syndromes and associations due to cross-reactivity between aeroallergens and food allergens of plant origin[9,11]

Syndrome or association (sensitivity to heat and proteases)	Relevant allergen components involved (allergen sources)
Birch-apple syndrome	Bet v 1 homologue Mal d 1
Cypress-peach syndrome	Pru p 3 non-specific lipid transfer protein (nsLTP)
Celery-mugwort-spice syndrome	Art v 4 profilin, Art v 60 kDa homologue to Api g 5
Mugwort-peach association	Art v 4 profilin, Art v 3 LTP
Mugwort-chamomile association	Art v 1 defensin (possible candidate)
Mugwort-mustard syndrome	Art v 3 LTP, Art v 4 profilin, Art v 60 kDa (possible candidates)
Ragweed-melon-banana association	Amb a 6 LTP, Amb a 8 profilin (possible candidates)
Goosefoot-melon association	Che a 2 profilin (possible candidate)

LTP: Lipid transfer protein.

judaica; *Amaranthaceae/Chenopodiaceae* family: rChe a 1, a 24 kDa trypsin inhibitor from the pollen of goosefoot *Chenopodium album* and nSal k 1, a 43 kDa protein belonging to the pectin methylesterase family from the pollen of saltwort *Salsola kali.* Art v 6 (pectate lyase) plays an important role in mugwort allergy and the cross-reactivity between Art v 6 and Amb a 1 is frequent, bidirectional, and clinically relevant.

Many cross-reactive allergen components are involved in pollen-food syndromes and associations, such as plant panallergen profilins (actin-binding proteins with roles in the dynamic turnover and restructuring of the actin cytoskeleton), PR-10 proteins (Bet v 1 homologues), lipid transfer proteins (LTPs)[9,27,33], as presented in Table 2.

Tree/shrub pollen aeroallergens and food allergens of plant origin

Several cross-reactivities between tree, shrubs and lianas pollen and foods are described in patients with respiratory allergy. Trees and shrubs discussed below as a source of pollen cross-reactive with foods belong to different anemophilous plant families: *Betulaceae* (birch family), *Oleaceae* (olive family), *Platanaceae* (plane-tree family), and *Cupressaceae (cypress family).* The temperate liana, vine *Vitis vinifera*, is also mentioned.

Regarding the birch-fruit-vegetable-syndrome, about 70% of birch pollen-allergic patients develop symptoms of allergy to plant foods, most frequently involved being *Rosaceae* fruits (mainly apple), nuts (especially hazelnut), and vegetables from the *Apiaceae* family (mainly celery and carrot). Pollinosis precedes the symptoms of food allergy, which, in the majority of cases, is limited to the oropharynx as oral allergy syndrome, occuring when eating raw food. The main allergen component involved in more than 90% of patients with birch pollinosis-associated food allergies, is Bet v 1, a pathogenesis-related PR-10 protein, which is cross-reactive with its homologous in these foods. Bet v 1 homologues represent major allergens in pollen of trees and shrubs from the order *Fagales* (including the *Betulaceae* and *Fagaceae* families), but can also be found in many allergenic foods belonging to the botanical families *Rosaceae* (50%-60% identity to Bet v 1 for the *Maleae* tribe PR-10 proteins: apple Mal d 1, pear Pyr c 1, and *Amigdaleae* tribe PR-10 proteins: apricot Pru ar 1, plum Pru c 1, peach Pru p 1, cherry Pru av 1), *Betulaceae* (hazelnut Cor a 1.0101 with 50% identity to Bet v 1), and *Apiaceae* (PR-10 proteins with 40%-50% identity to Bet v 1: carrot Dau c 1, celery Api g 1). Less than 25% of patients with this syndrome are sensitized to the panallergen Bet v 2 (birch profilin), its contribution to symptoms being unclear. Bet v 1 homologues and profilins, incriminated in the birch-plant foods syndrome, are denatured by high temperatures and by gastric enzymes[20,25,34-37].

The birch pollen-hazelnut association is a *Betula* pollen-associated food allergy to hazelnuts, with Cor a 1-reactive T cells and specific IgE cross-reactive to Bet v 1[38]. This type of hazelnut (*Corylus avellana*) allergy occurs in adults with pollinosis to *Betulaceae* trees/shrubs, and manifests mainly as an oral allergy syndrome, due to an extensive cross-reactivity between the labile hazelnut Cor a 1.04 and birch pollen Bet v 1 allergen components. In contrast, children predominantly exhibit sensitisation to hazelnut storage proteins, Cor a 9 and Cor a 11, which is unrelated to birch pollen allergy, and had more severe clinical manifestations on consumption on raw and processed hazelnuts. In the absence of a cure, avoidance remains the key measure of effective management, especially in patients with severe symptoms[39].

The most common tree pollen-fruit cross-reactivity is represented by the birch-apple syndrome[11,40]. IgE antibodies formed against either Bet v 1, the birch pollen PR-10 allergen, or Mal d 1, the apple allergen, cross-react and give rise to sensitivity to both birch and apple. Moreover, patients with oral allergy syndrome to apple have a higher Bet v 1-induced T cell proliferation compared with those monosensitized to birch pollen without food allergy[12,41].

Interestingly, soy allergen component Gly m 4 also belongs to the PR-10 protein family, and in birch pollen-allergic patients, the combination of IgE sensitization to Gly m 4 and intake of large amounts of mildly processed soy, like soy drinks, may induce a severe allergic reaction[12].

The birch-apple-carrot association is another possible cross-reaction in patients with birch pollen and food allergy, in which IgE-mediated systemic allergic reaction to both apple and carrot, in both fresh and cooked form, is reported[42]. The birch-*Apiaceae* syndrome is seen

mainly in central Europe, and the typical clinical picture is oral allergy syndrome, which occurs when raw foods are ingested. This food allergy to *Apiaceae* is secondary to pollinosis and is due to the presence in these foods of Bet v 1 homologues (Api g 1, Dau c 1), and less frequently to profilins[34].

IgE sensitization patterns to different cross-reactive allergen components are variable according to climate and eating habits. In the Western Mediterranean region, allergies to *Rosaceae* fruits are caused by monosensitization to profilin, monosensitization to LTP, or co-sensitization to both these allergen molecules. In Northern and Central Europe, monosensitization to PR-10 and, to a lesser degree, co-sensitization to profilin and PR-10, is dominant. LTP sensitization is present both in pollinosis and non-pollinosis patients, and is associated with peach allergy in particular. The disease pattern for patients sensitized to profilin is characterized by several concomitant allergies, including grass pollen, *Rosaceae* and non-*Rosaceae* fruits. Sensitization to PR-10 is primarily associated to concomitant birch pollen and apple allergy[43]. In a birch endemic area in Western Europe, both mild and anaphylactic apple-allergic patients are sensitized to PR-10 proteins, whereas only a few of the mild local and none of the anaphylactic apple-allergic patients is sensitized to LTP. In contrast, anaphylactic hazelnut-allergic patients display no such clear sensitization pattern: few are sensitized to both PR-10 proteins and hazelnut LTP, and others to only LTP or to only PR-10 proteins, or to neither PR-10 proteins, nor LTP[44]. Bet v 1 sensitization is associated to concomitant birch pollen rhinoconjunctivitis and oral allergy syndrome to *Rosaceae* fruits in patients from the Southeastern-Central Europe, in a sylvosteppe area with low density forests[45]. In East-Central Europe, in patients with birch pollen allergy with associated food allergy, IgE sensitization to Bet v 1 is frequently associated with food allergy to fruits from *Rosaceae* family. Bet v 2 profilin may be involved in cross-reactivity with non-*Rosaceae* plants, such as *Apiaceae*/*Umbelliferae* vegetables[46].

Immune tolerance induction in the birch-apple syndrome was evaluated in several studies. In patients with oral allergy to apple, tolerance can be safely induced with slowly, gradually increasing consumption of apple, but relapse after consumption discounting and absence of immunologic changes suggest it is only transient[47]. Allergy immunotherapy is clearly effective for birch pollen allergy, but its efficacy on apple allergy is still controversial. Some patients treated with subcutaneous or sublingual immunotherapy develop complete tolerance to apple. Pre-treatment evaluation of patients using molecular allergy diagnosis tools and choosing the appropriate immunotherapeutical doses of birch pollen allergen extract is important[40,48]. Although most patients became re-sensitized to apple over time, many of them are still able to tolerate eating apple at a 30-mo follow-up visit[49]. Pollen immunotherapy has also a positive impact on oral allergy syndrome to hazelnut in birch pollen-allergic patients, but the amount of hazelnut tolerated is small, the effect remaining limited[50].

In the cross-reactive olive pollen-fruit syndrome the main fruits involved are peach *Prunus persica*, pear *Pyrus communis*, melon *Cucumis melo* and kiwi fruit *Actinidia deliciosa*. Sensitization to the LTP Ole e 7 is associated with more severe clinical symptoms in patients who had anaphylaxis, while to the profilin Ole e 2 in most oral allergy syndrome cases. Cross-reactivities between profilins (Ole e 2, Pru p 4, Pyr c 4, Cuc m 2, Act d 9) and LTPs (Ole e 7, Pru p 3, Pyr c 3, Cuc m LTP, Act d 10) are involved in the olive pollen–fruit syndrome. The glucanase Ole e 9 is an allergen component candidate for an important role in pollen-latex-fruit syndrome in patients allergic to olive pollen. Beta-glucanases are also present in latex *Hevea brasiliensis* (Hev b 2) and banana (Mus xp 5). Other cross-reactive allergens involved in pollen-latex-fruit syndrome are profilins from olive pollen (Ole e 2), latex (Hev b 8), ananas (Ana c 1), banana (Mus xp 1) and kiwi (Act d 9), and superoxide dismutases from olive pollen (Ole e 5) and latex (Hev b 10)[51].

In the ficus-fruit syndrome, allergic reactions to fresh or dried figs (*Ficus carica*) or other tropical fruits, which may be presented as anaphylaxis, are a consequence of primary sensitization to airborne ornamental *Ficus benjamina* allergens, independent of sensitization to rubber latex allergens. Cross-reactive ficus allergen component ficin (Fic c Ficin) belongs to the family of cystein proteases present also in kiwi fruit *Actinidia deliciosa* (Act d 1), pineapple *Ananas comosus* (Ana c 2), papaya *Carica papaya* (Car p 1). Figs may also be involved in the latex-fruit syndrome[52,53].

The *Platanus* pollen-fruit/vegetables association is a cross-reactivity entity observed among plane tree *Platanus acerifolia* pollen and plant-derived food allergic patients. There is an important cross-reactivity between the pollen of plane tree, hazelnut and banana fruit, and an intermediate cross-reactivity with celery and peanut. Other fruits and vegetables may also be mentioned. The cross-reacting LTPs may be involved, being present in *Platanus acerifolia* pollen (Pla a 3), but also in hazelnut *Corylus avellana* (Cor a 8), banana *Musa acuminata* (Mus a 3) fruit, peach (Pru p 3), celery *Apium graveolans* (Api g 2), peanut *Arachis hypogaea* (Ara h 9). It appears that neither profilin Pla a 8, nor the two major allergens invertase inhibitor Pla a 1 and polygalacturonase Pla a 2 can be the cause for the strong cross-reactivity[54-57].

In the cypress-peach syndrome, allergic crossreactions between cypress pollen and peach have been reported, including oral allergy syndrome. Profilins (Cup s 8, Pru p 4) or thaumatins (Cup s 3, Pru p 2) could not explain the observed clinical association between cypress pollen and peach. Pru p 3-like non-specific LTPs are involved in the syndrome[11,58].

Other rarer cross-reactive associations are those between date-palm (*Phoenix dactylifera*) pollen and vegetal foods manifested as oral allergy syndrome[59] and between *Vitis vinifera* vine pollen eliciting seasonal

rhinoconjunctivitis and asthma with subsequent food allergy to grapes[60], in both the cross-reacting proteins are still not well established.

Grass pollen aeroallergens and food allergens of plant origin

Profilins are highly cross-reactive allergen components which bind IgE antibodies of almost 20% of plant-allergic patients. Grass pollen is cross-reactive with some foods in patients with oral allergy syndrome. The Bermuda grass *Cynodon dactylon* pollen profilin (Cyn d 12) has substantial cross-reactivity with profilins from tomato *Solanum lycopersicum* (Sola l 1) and cantaloupe *Cucumis melo* (Cuc m 2)[3,61].

Although several patients with oral allergy syndrome, urticaria, angioedema, gastrointestinal or anaphylaxis symptoms after ingestion of products containing wheat or maize flour were reported in patients suffering from respiratory allergy to grass pollens, cross-reactivity among cereal grains and grass pollen is generally considered clinically insignificant. Beta-expansin 11 (EXPB11), a homologue of the major allergen of timothy grass pollen, Phl p 1, may bear a high cross-reactive potential in patients who suffer from both food allergy and pollinosis[62,63].

Hypersensitivity reactions to Navajo ceremonial use of oral corn pollen in native Americans were previously described[64], and recently a case of corn silk (*Stigma maydis*) infusion (traditional herbal medicinal product) and *Poaceae* pollen allergy was reported[65].

Weed pollen aeroallergens and food allergens of plant origin

Allergenic weeds with pollen involved in respiratory sensitization followed by food allergy due to cross-reactive allergen components can be found in the plant families of *Asteraceae/Compositae* (mugwort *Artemisia vulgaris*, ragweed *Ambrosia artemisiifolia* var. *elatior*), *Urticaceae* (pellitory *Parietaria officinalis*), *Amaranthaceae/Chenopodiaceae* (goosefoot *Chenopodium album),* *Plantaginaceae* (plantain *Plantago lanceolata*), and *Cannabaceae* (hop *Humulus japonicus*)[9,66].

In the celery-birch-mugwort-spices syndrome, patients IgE-sensitized to *Betula* and *Artemisia* spp pollen present food allergy to celery, other vegetables and spices, due to several cross-reactive allergen components, including Bet v 1 homologs, profilins and high molecular weight allergens of 40-60 kDa. The Bet v 1 homologs, Api g 1 (celery) and Dau c 1 (carrot), are responsible for the association between birch pollinosis and *Apiaceae* allergy, as no Bet v 1-homologous proteins are found in mugwort pollen. Thus, the celery-birch association only involves species from the *Apiaceae* family, whereas the celery-birch-mugwort syndrome comprises additional botanical families. Associated allergy to *Amaryllidaceae* family foods and spices is rare[9,34].

The well-known celery-mugwort-spice syndrome consists of respiratory sensitization to mugwort *Artemisia vulgaris* and IgE cross-reactive reactions to foods belonging to *Apiaceae/Umbelliferae* family: celery (*Apium graveolans*), carrot (*Daucus carota*), parsley (*Petroselinum crispum*), caraway seeds (*Carum carvi*), fennel seeds (*Foeniculum vulgare*), coriander seeds (*Corinadrum sativum*), aniseed (*Pimpinella anisum*); *Amaryllidaceae* family: garlic (*Allium sativum*), onion (*Allium cepa*), leek (*Allium porrum*); *Solanaceae* family: paprika (*Capsicum annuum*); and *Piperaceae* family: pepper (*Piper* sp). The number of allergen sources involved, the nature of the allergen components, and influencing factors, such as climate and dietary habits, make the celery-birch-mugwort-spice syndrome a clinical entity of high complexity[9]. Complicated cases may associate curry spice allergy with pollen-food allergy syndrome and latex fruit-syndrome[67]. Cross-reactivity between profilins of mugwort pollen (Art v 4) and *Apiaceae* foods, such as celery (Api g 4), carrot (Dau c 4) and spices, are involved in the pathogenesis of this celery-mugwort-spice syndrome[9]. Moreover, the mugwort-fennel-allergy-syndrome is associated with sensitization to an allergen homologous to Api g 5, a high molecular weight glycoprotein flavoprotein, Foe v 5, with function of *flavin adenine dinucleotide-linked oxidoreductase*. An Api g 5-like protein was also identified in carrot. This 60 kDa fennel allergen, highly homologous to Api g 5, may be involved in the mugwort-celery-spice syndrome, being cross-reactive with Art v 60 kDa allergen component from mugwort pollen[68,69].

The mugwort-mustard allergy syndrome describes the association of mugwort pollinosis with several botanically unrelated plant-derived foods allergy from the *Brassicaceae/Cruciferae* family: white mustard (*Sinapis alba*), Indian mustard (*Brassica juncea*), cabbage (*Brassica oleracea* var. *capitata*), broccoli (*Brassica oleracea* var. *italica*), cauliflower (*Brassica oleracea* var. *botrytis*), and possibly from the *Fabaceae/Leguminosae* family: peanut (*Arachis hypogaea*), and *Rosaceae* family: almond (*Prunus dulcis*). Mustard is sometimes a masked allergen in processed foods, and food allergy symptoms vary from oral allergy syndrome to anaphylaxis. Although the causative cross-reactive allergen components have not yet been clearly identified, the possible candidates are LTPs (Art v 3, Sin a 3), profilins (Art v 4, Sin a 4) and high molecular weight allergens, such as Art v 60 kDa[9].

In the mugwort-peach association, the cross-reactive allergen components involved are LTPs (Art v 3, Pru p 3) and profilins (Art v 4, Pru p 4)[9]. This cross-reactivity association appears in a limited group of patients from Southern Europe, in which Art v 3 behaves as the primary sensitizing allergen, although Pru p 3-associated peach allergy is a food allergy driven by primary sensitization to peach in the Mediterranean region. The *Rosaceae* fruits allergens cross-react with mugwort allergens in the mugwort-peach association[70]. A similar group of patients was reported recently in Northern China, in which the food peach LTP allergy originates

from primary sensitization to cross-reactive pollen allergen component Art v 3. The pattern of geographical distribution of this mugwort-peach association may be explained by the dominant role of mugwort pollen exposure in some regions, which is similar to the importance of birch pollen exposure in Northern Europe[71].

An *Asteraceae*-lychee association was also reported in patients diagnosed with respiratory allergy to *Artemisia vulgaris* pollen and food allergy to sunflower seeds, who have subsequently presented anaphylaxis after the first ingestion of lychee fruit. *Litchi chinensis* is a tropical fruit belonging to the *Sapindaceae* family, in which Lit c 1 profilin, a 16 kDa allergen cross-reactive with Art v 4 profilin, was identified. Another allergen component of 70 kDa identified in lychee, and also present in mugwort pollen, is a possible new candidate for the cross-reactive mechanism in this clinical association[72].

In mugwort-chamomile association, respiratory IgE sensitization to mugwort *Artemisia vulgaris* is a primary risk factor for allergy symptoms up to anaphylaxis to ingestion of chamomile infusions. Some patients present positive conjunctival provocation tests with chamomile extract. German Chamomile (*Matricaria chamomilla* var. *recutita*) is a Southern European plant of the *Asteraceae* family, used frequently as herbal tea medical remedy. There is a high degree cross-reactivity between *Matricaria chamomilla* and *Artemisia vulgaris* pollen[73]. The candidate cross-reactive component proposed in mugwort-chamomile association is the Art v 1 defensin[11]. Bet v 1 homologues (Mat c 1) and high molecular weight allergens may also play a role, but not profilins. Patients sensitised to mugwort pollen sometimes present allergic reactions to chamomile, while most subjects allergic to chamomile are sensitized to mugwort. In clinical practice, the incidence and risks of this mugwort-chamomile association may be underestimated[74,75].

In mugwort-sunflower association, the food allergy was reported either manifested as anaphylaxis to consumption of sunflower-pollen contaminated commercial peeled sunflower seeds in a patient sensitized to *Artemisia* pollen, or as oral allergy syndrome after eating sunflower seeds in a patient with airborne allergy to particles of these seeds used as pet food for small animals, such as birds. Moreover, allergy to sunflower seeds may be associated with respiratory allergy to mugwort pollen[76]. Sunflower (*Helianthus annuus*) belongs to the family of *Asteraceae*. Its pollen allergen component Hel a 4 is an Art v 1-like allergen, while Hel a 3 LTP and Hel a 2S albumin are present in seeds[77-79].

Clinical cases of severe allergic reactions, mostly anaphylaxis, to ingestion of bee products, containing pollen grains of *Compositae* plants, in patients with respiratory allergy to *Asteraceae* pollen, especially mugwort and ragweed pollen, were published, for honey, royal jelly and bee polen dietary supplements[80-83]. Cross-reactivity between the pollen of wind-pollinated weeds and other *Asteraceae* insect-pollinated plants is a major mechanism of bee product-induced allergic reactions, likely attributable to several contained pollen allergen components, including profilins, polcalcins, and LTPs. Bee products may contain not only pollen from insect-pollinated plants, but also from wind-pollinated trees or herbaceous plants that grow in the same area, resulting in systemic allergic reactions after accidental ingestion of these airborne pollen grains[81].

The ragweed-melon-banana association is present in patients with respiratory allergy to ragweed *Ambrosia artemisiifolia* experiencing food allergy, usually oral symptoms, when eating various members of the *Cucurbitaceae* family: watermelon (*Citrullus lanatus* subsp. *vulgaris*), netted muskmelon/cantaloupe (*Cucumis melo* var. *cantalupo*), honeydew melon (*Cucumis melo* var. *inodorus*), zucchini (*Cucurbita pepo*), cucumber (*Cucumis sativus*); and *Musaceae* family: banana (*Musa* x *paradisiaca*) fruit. The possible cross-reactive allergen candidates involved in ragweed-melon-banana association are profilins (Amb a 8, Cit la 2, Cuc m 2, Cuc p 2, Cuc s 2, Mus xp 1) and LTPs (Amb a 6, Cuc m LTP)[9,84]. Another possible cross-reactivity between *Ambrosia* pollen and other plants from the *Asteraceae* family recommends not to administer *Echinacea* botanical supplements in patients allergic to ragweeed[85].

In the pellittory-pistachio association, several cross-reacting proteins were suggested using *in vitro* methods. IgE sensitization to pistachio (*Pistacia vera*) is common in *Parietaria* weed pollen allergy. Pistachio nuts, belonging to the family of *Anacardiaceae*, are widely used to produce ice creams, cakes, and mortadella, or are simply eaten roasted. Oral allergy syndrome to pistachio was reported in adult and child patients with *Parietaria* pollinosis. Minor injuries of the oral mucosa due to pistachio shells may enhance local allergic responses[9,86]. Furthermore, different members of the *Anacardiaceae* family (pistachio, mango, and cashew) have been mentioned to share common allergens[87].

The plantain-melon association represents the clustering of allergy to melon *Cucumis melo* and plantain *Plantago lanceolata*, respiratory sensitization to plantain pollen being important in Australia and Mediterranean countries. Several distinct proteins of 14 and 31 kDa, and a spectrum of proteins migrating between 40 and 70 kDa were discussed as cross-reactive allergens[9]. Moreover, the seeds of *Plantago ovata* (psyllium, ispaghula) used in the manufacture of bulk laxatives may cause occupational respiratory allergy in health care and pharmaceutical workers. Cases of anaphylaxis were reported after ingestion of laxatives or breakfast cereals containing *Plantago ovata* seeds, in most of those subjects sensitization occurring previously by inhalation of seed dust. In addition, immunologic cross-reactivity between *Plantago ovata* seed and *Plantago lanceolata* pollen is possible[88].

The goosefoot-melon association or the goosefoot-fruit association was revealed in several cases of patients with pollen allergy to *Chenopodium album*

Table 3 Examples of syndromes due to cross-reactivity between aeroallergens of animal and fungal origin and food allergens[9,11]

Syndrome or association	Relevant allergen components involved
Alternaria-spinach syndrome	Al t a 1
Mite-shrimp syndrome	Der p 10 tropomyosin
Cat-pork syndrome	Fel d 2 cat serum albumin
Bird-egg syndrome	Gal d 5 alpha-livetin (chicken serum albumin)

(allergenic weed of the *Amaranthaceae/Chenopodiaceae* family), who displayed oral allergy syndrome after eating fresh fruits, such as melon, banana and peach. The panallergen profilins (Che a 2, Mus xp 1, Pru p 4) might play a role in goosefoot IgE cross-reactivity[9]. The risk for Russian thistle-saffron association is due to the possible cross-reactivity between *Salsola* and saffron, keeping in mind the use of saffron as a *Crocus sativus* flower-derived spice and the reported flower sensitization in saffron workers[9].

Finally, a hop-celery association is mentioned for Japanese hop (*Humulus japonicus*) pollinosis with allergy to another representative of the *Cannabaceae* family, the common hop (*Humulus lupulus*), and to the unrelated celery (*Apium graveolans*), with no significant associations with ragweed or mugwort pollen[9,89].

CROSS-REACTIVITY BETWEEN AEROALLERGENS OF FUNGAL ORIGIN AND FOOD ALLERGENS

Respiratory allergy to environmental molds is relatively common, fungi representing a prominent source of aeroallergens[90]. *Alternaria alternata* is one of the most common molds associated with allergic diseases, and 80% of *Alternaria*-sensitive patients produce IgE antibodies to Alt a 1, a major allergen with an unique, dimeric beta-barrel structure. Alt a 1 and homologous proteins are characteristic for the *Dothideomycetes* class of ascomycetes[91].

The *Alternaria*-spinach syndrome, in which Alt a 1 is mentioned as an involved allergen component (Table 3), was recently recognized[11]. This cross-reactivity between aeroallergens from fungi imperfecti and allergens from spinach and mushroom *Agaricus bisporus* as foods is mentioned. In the first report of anaphylaxis to spinach and concomitant oral allergy syndrome to mushrooms, cross-reactivity was suggested to be due to common epitopes[92]. A further study identified the cross-reactive 30 kDa protein, probably the Alt a 1 allergen component, present both in spinach and mushroom extracts[93]. This syndrome is different from another possible association between IgE sensitization to mannitol, naturally present in cultivated mushrooms and pomegranate, and anaphylaxis to this sugar alcohol as a drug excipient[94].

In addition, non-fatal anaphylaxis was reported after mycoprotein burger eating, in a young female patient with respiratory IgE-allergy to *Alternaria alternata*, the edible mycoprotein being produced from the fungus *Fusarium venenatum*. Cross-reactivity studies revealed that it shares allergenic determinants with *Alternaria alternata* and *Cladosporium herbarum*[95]. Sensitization to mold allergens *via* the respiratory tract and subsequent oral ingestion of cross-reactive fungal proteins may lead to severe food-allergic reactions, such as those caused by *Fusarium venenatum* acidic ribosomal protein P2 allergen[96]. An unusual case of fatal anaphylaxis was also reported due to heavy mold contamination of a pancake mix with *Fusarium, Penicillium*, *Mucor*, and *Aspergillus* spp, in a teenager allergic to molds[97]. It is also possible that bee products, such as bee pollen supplements, to be contaminated with fungi such as *Aspergillus* and *Cladosporium* spp, and may cause severe allergic reactions in patients sensitized to these molds[81].

Moreover, yeasts should be considered as possible ingestive allergens in mold-allergic patients. A patient with a clustered respiratory IgE sensitization to fungi (*Alternaria alternata*, *Cladosporium herbarum*, *Aspergillus fumigatus*, and *Penicillium notatum*) and baker's yeast (*Saccharomyces cerevisiae*), developed multiple anaphylactic reactions after ingesting pasta yeast sauces containing cross-reacting fungal allergens[98].

CROSS-REACTIVITY BETWEEN AEROALLERGENS OF ANIMAL ORIGIN AND FOOD ALLERGENS

Aeroallergens of animal origin, such as those from domestic arthropods (house dust mites, cockroaches) and pets are usual indoor allergens involved in allergic rhinitis and asthma, along with indoor fungal aeroallergens from certain molds. Spending more time in areas inside buildings (homes, workplaces, schools, and indoor public spaces) creates conditions for more exposure to multiple indoor aeroallergens[99]. Examples of syndromes due to cross-reactivity between inhaled allergens of animal origin and food allergens are presented in Table 3. Other data from case reports defining rare or distinct associations are also discussed below, but are not simplistic put in the table as general rule for clinical practice.

Aeroallergens and food allergens of invertebrate animal origin

Dust mites are the most common indoor environmental cause of respiratory allergies. The main sources of allergens in house dust worldwide are the mite species belonging to the phylum of *Arthropoda*, class *Arachnida*, subclass *Acari*), order *Astigmata*. The most important house dust mites are those from the *Pyroglyphidae* family, especially *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*. Pyroglyphid mites are highly cross-reactive, but have limited cross-reactivity with *Blomia tropicalis*, mite species from *Echimyopodidae*

family significant for tropical/subtropical regions, or with other storage mites from *Acaridae* and *Glycyphagidae* families[100,101].

Patients sensitized to house dust mites can be classified according to their pattern of sensitization into those sensitized only to the major allergens from group 1 and group 2 allergens of pyroglyphid mites, and those with a broader profile of sensitization, including highly cross-reactive allergens, the most important being tropomyosin, belonging to the group 10 allergens[102].

Allergic reactions to edible invertebrates can generate a variety of clinical manifestations ranging from mild oral allergy syndrome, urticaria and/or angioedema, to severe anaphylaxis. Although cross-reactivity between dust mites and invertebrates consumed as food is demonstrated, sometimes there is a poor correlation of IgE reactivity and clinical symptoms[103].

Edible invertebrates are represented mostly by shellfish (culinary term for exoskeleton-bearing aquatic invertebrates, such as crustaceans and mollusks) and less by edible insects (in some parts of the world). Although most kinds of shellfish are aquatic invertebrate animals from saltwater environments (seafood), some are harvested from freshwater, and, in addition, few species of land snails and crabs are also edible invertebrates. Crustaceans are classified among arthropods, together with arachnids (including house dust mites) and insects, whereas mollusks include bivalves, cephalopods and gastropods. Shellfish is one of the leading causes of food allergy in adults and is a common cause of food-induced anaphylaxis. Most frequent causative types of shellfish are shrimp, crab, lobster, clam, oyster and mussel. Specific invertebrate seafood allergy can reflect regional consumption of particular species. It is also important to differentiate shellfish toxic syndromes frequently masquerading as an allergic reaction[104].

The house dust mites-crustaceans-mollusks syndrome is a relatively rare variant of food allergy in which the house dust mites are the primary IgE sensitising agents, while shellfish can induce food allergy, up to anaphylaxis, even at first ingestion[23]. In the more usual mite-shrimp syndrome, the typical allergen component mentioned is tropomyosin Der p 10[11]. Allergenic molecules involved in shellfish allergy are cross-reactive with allergen components from house dust mites, especially certain proteins with a role in muscular contraction. Cross-reactivity with species that are not closely related is common in shellfish-allergic patients, some seafood allergens being widely distributed invertebrate panallergens[105]. Tropomyosin was the first identified allergen involved in cross-reactivity between *Dermatophagoides pteronyssinus* mite, shrimp and insects[106] and it is still considered a major shellfish allergen frequently responsible for clinical cross-reactivity with inhaled house dust mites[107]. Besides been assumed to be a major cause of cross-reactivity between astigmatid mites and other invertebrates, tropomyosin may be a major cause of covariation of sensitization between house dust mites, crustaceans, and some species of insects and mollusks[22]. Allergenic tropomyosins are highly conserved muscle proteins found in invertebrates, such as arachnids (house dust mites), insects (cockroaches), crustaceans (shrimp, prawn, lobster, crawfish, crab), and mollusks (mussel, oyster, squid, cuttlefish, octopus, abalone, limpet, snail), therefore being considered panallergens. In contrast to invertebrate tropomyosin, vertebrate tropomyosins, such as those from beef, pork, rabbit or chicken, are not allergenic[104,108].

Tropomyosins are a large family of alpha-helical proteins that form a coiled-coil structure of two parallel helices containing two sets of seven alternating actin-binding sites, playing a critical role in regulating the function of actin filaments. Tropomyosins are present in all eukaryotic cells, associated with the thin filament in muscle and microfilament in many nonmuscle cells, being involved in the contractile activity of these cells, and also in helping regulation of cell morphology and motility. As panallergens, these are resistant to heat, low gastric pH and gastroenteric peptidase, therefore their allergenicity is maintained in cooked and digested foods, causing allergic systemic reactions up to anaphylaxis. Natural tropomyosin has an average molecular weight of 37 kDa. House dust mite group 10 allergens are composed of 284 amino acids. Sequence identity within the house dust mite tropomyosins is higher than any other mite allergen. The Der p 10 tropomyosin shares more than 65% identical residues with other invertebrate tropomyosins[22,109-111]. Regions adjacent to the positions 133-135 and 201 of the invertebrate tropomyosins present lower probability of alpha helix folding than those of vertebrates, and are candidates responsible for allergenicity[112]. There is a high cross-reactivity between house dust mite tropomyosins: *Dermatophagoides pteronyssinus* (Der p 10), *Dermatophagoides farinae* (Der f 10) and *Blomia tropicalis* (Blo t 10)[22].

The prevalence of sensitization to tropomyosin among house dust mite-allergic patients, assessed using recombinant group 10 mite allergen components, varies with geographical area[22,113]. In many European countries, this sensitization prevalence to rDer p 10 varies between 9%-18%[114]. Other studies revealed that sensitization rates to tropomyosin are found in higher rates (30%-55%) in subtropical or tropical regions, such as Australia or Central Africa. This variability can be explained by various exposure to sensitizing invertebrate allergens in different parts of the world[113]. The resulting IgE antibodies are able to cross-react with different tropomyosins, even with those which did not induce their production. For example, IgE antibody reactivity to a major food allergen, the cross-reactive brown shrimp tropomyosin, can occur in unexposed subjects (Orthodox Jews, with Kosher dietary laws that prohibit eating shellfish) with clinically significant allergy to house dust mites and/or cockroaches[21]. Another study revealed that half of the house dust mite-allergic

European patients with IgE sensitization to tropomyosin (Der p 10) have a history of clinically relevant cross-reactivity reactions to eating seafood, the other half having no allergic reactions when consuming such edible invertebrates[107].

Cross-reactivity of tropomyosin allergens from house dust mites with crustaceans (subphylum *Crustacea*) is significant, mentioning decapods (order *Decapoda*) from the *Penaeidae* family: brown shrimp *Farfantepenaeus/Penaeus aztecus* (Pen a 1), black tiger shrimp *Penaeus monodon* (Pen m 1), Indian prawn *Fenneropenaeus/Penaeus indicus* (Pen i 1), whiteleg shrimp *Litopenaeus vannamei* (Lit v 1), sand shrimp *Metapenaeus ensis* (Met e 1); *Crangonidae* family: common shrimp *Crangon crangon* (Cra c 1); *Pandalidae* family: Northern shrimp *Pandalus borealis* (Pan b 1); *Nephropidae* family: European lobster *Homarus gammarus* (Hom g 1), American lobster *Homarus americanus* (Hom a 1); *Palinuridae* family: Chinese spiny lobster *Panulirus stimpsoni* (Pan s 1); *Portunidae* family: coral crab *Charybdis feriata* (Cha f 1), and *Cambaridae* family: red swamp crayfish *Procambarus clarkii (*Pro cl 1)[22,104,115]. Cross-reactivity of tropomyosin allergens from house dust mites with mollusks (phylum *Mollusca*) is also reported, mentioning *Bivalvia* class mussels (*Mytilidae* family): blue mussel *Mytilus edulis* (Myt e 1), Mediterranean mussel Mytilus galloprovincialis (Myt g 1), Asian green mussel Perna viridis (Per v 1), oysters (Osteridae family): Pacific oyster *Crassostrea gigas* (Cra g 1), scallops (*Pectinidae* family): scallop *Mimachlamys nobilis* (Mim n 1), razor clams (*Solecurtidae* family): constricted tagelus *Sinonovacula constricta* (Sin c 1); *Gastropoda* class abalones (*Haliotidae* family): disk abalone *Haliotis discus hannai* (Hal di 1), Japanese abalone *Haliotis diversicolor* (Hal d 1), turban snails (*Turbinidae* family): horned turban *Turbo cornutus* (Tur c 1), land snails (*Helicidae* family): brown garden snail *Helix aspersa* (Hel as 1); *Cephalopoda* class decapod arrow squids (*Ommastrephidae* family): Japanese flying squid *Todarodes pacificus* (Tod p 1), cuttlefish (*Sepiidae* family): golden cuttlefish *Sepia esculenta* (Sep e 1), and octopods (*Octopodidae* family): common octopus *Octopus vulgaris* (Oct v 1)[22,104,115,116]. In addition to tropomyosin, other muscle protein crustacean allergens involved in shellfish allergy and cross-reactivity with other invertebrates are arginine kinase and myosin light chain[117].

Arginine kinase, a 40-kDa enzyme involved in the storage of excess energy as arginine phosphate, is a potential new class of invertebrate panallergens, identified mainly in crustaceans, such as black tiger shrimp *Penaeus monodon* (Pen m 2), common shrimp *Crangon crangon* (Cra c 2), whiteleg shrimp *Litopenaeus vannamei* (Lit v 2), Chinese shrimp *Fenneropenaeus chinensis* (Fen c 2), snow crab *Chionoecetes opilio* (Chi o 2), mangrove mud crab *Scylla serrata* (Scy s 2), Atlantic Horseshoe Crab *Limulus polyphemus* (Lim p 2), but also in mollusks: common octopus *Octopus vulgaris* (Oct v 2), ocellated octopus *Octopus fangsiao* (Oct f 2). These are cross-reactive with arginin kinase allergens from house dust mites (Der p 20, Der f 20, Blo t 20), cockroaches (Bla g 9, Per a 9) and moths, such as Indian-meal moth *Plodia interpunctella* (Plo i 1) and silk moth/silkworm larvae *Bombyx mori* (Bomb m 1)[104,118-121].

Myosin light chain is a 20 kDa crustacean allergen identified in common shrimp *Crangon crangon* (Cra c 5), brine shrimp *Artemia franciscana* (Art fr 5), black tiger shrimp *Penaeus monodon* (Pen m 3), whiteleg shrimp *Litopenaeus vannamei* (Lit v 3), American lobster *Homarus americanus* (Hom a 3), with potential cross-reactivity with aeroallergens from dust mite *Dermatophagoides farinae* (Der f 26) and German cockroach (Bla g 8)[104,120,122,123].

Paramyosin is a 103 kDa cross-rective muscle protein described in diverse invertebrates. Molluskan paramyosin is responsible for the "catch" mechanism that enables sustained contraction of muscles with very little energy expenditure. House dust mite allergen Der p 11 (paramyosin with 89% identity with Der f 11) presents significant homology with the paramyosin of mollusks: Mediterranean black Mussel *Mytilus galloprovincialis* (Myt g PM), Japanese Abalone *Haliotis discus* (Hal di PM), Horned Turban *Turbo cornutus* (Tur c PM), common Octopus *Octopus vulgaris* (Oct v PM)[116,120,124,125].

Amylase and haemocyanin are other allergens found in mollusks and are possibly involved in cross-reactivity with house-dust mite aeroallergens. Alpha-amylase Der p 4 may be involved in *Dermatophagoides pteronyssinus* cross-reactivity in gastropod allergy[22,116,126].

It is important to differentiate primary sensitization to pyroglyphid mite aeroallergens, with subsequent food allergy to edible invertebrates, from allergy to shellfish in patients not allergic to house dust mites. There is a profile of sensitization to shellfish in which tropomyosin is involved as a panallergen, with patients not tolerating several crustaceans and/or mollusks, selective sensitization to only one type of seafood being uncommon. Another profile has been also described in cases with house dust mites as primary sensitizing agents and selective allergy to mollusks or crustaceans, described for common European limpet (*Patella vulgata*), terrestrial green garden snail (*Helix* spp) and Mediterranean spiny lobster (*Palinurus elephas*)[126,127]. The role of tropomyosin as a clear cause of cross-reactivity is a matter of debate in some circumstances. Clinical cases of shrimp allergy without snail allergy in relation to house dust mites sensitization, and of allergy to snails without shrimp allergy in context of respiratory mite allergies, were reported, with the observation that in shrimp allergy the symptoms are mainly urticaria or angioedema, while in snail allergy the clinical picture is usually dominated by severe asthma[128].

Moreover, there seems to exist differences in mite-shellfish cross-reactivity depending on climate. A recent study designed to identify which of the shrimp allergen molecules (tropomyosin, arginine kinase, sarcoplasmic

calcium-binding protein, actinins, aldolase, ubiquitin) are involved in mite-seafood cross-reactivity in two different climate populations, revealed that tropomyosin and ubiquitin are responsible for mite-seafood cross-reactivity from both continental dry and humid climates, while alpha-actinin and arginine kinase are involved in dry- and humid-climate groups, respectively. Mites are the primary sensitizer in humid-climate, while shrimps in the continental dry-climate population[129]. Patients sensitized to tropomyosin Der p 10 usually are sensitized to several other house dust mite allergen components besides the major allergens (Der p 1, Der p 2), whereas Der p 10-negative patients are primarily sensitized to Der p 1 and/or Der p 2. Therefore, Der p 10 may be a diagnostic biomarker for mite-allergic patients with additional sensitization to allergens other than Der p 1 and Der p 2, such patients requiring more attention when immunotherapy with allergen extracts is considered[130].

Because allergy immunotherapy with house dust mites extracts is an effective method of treating respiratory allergy, and most of the currently available extracts for subcutaneous or sublingual route of administration contain high concentrations of group 1 and 2 allergens, but may also contain lower concentrations of other sensitizing molecules, including group 10 allergens, it is still unclear whether this type of treatment may induce clinically relevant sensitization to tropomyosin[113,131]. Some studies suggested that food allergy to shrimp or snail can worsen in some patients treated with subcutaneous immunotherapy. However, many patients already had mild allergic reactions to tropomyosin-containing foods before starting immunotherapy, and a new sensitization was confirmed in only one patient[132,133]. Other studies revealed a lack of induction of new sensitization to tropomyosin during house dust mite injection or sublingual immunotherapy[131,134]. Additional studies suggested that, apart from not inducing new sensitization to tropomyosin, house mite subcutaneous immunotherapy could have possible beneficial effects in patients with snail, squid or shrimp allergy[113,135]. Moreover, a recently published report presented a shrimp allergy case improved from anaphylactic symptoms observed before, to mild oral allergy syndrome after one year of sublingual mite immunotherapy with a known and relatively high dosage of tropomyosin[136]. Therefore, induction of clinically relevant sensitization to tropomyosin is an unlikely consequence of house dust mite immunotherapy, but since the risk of adverse allergic reactions to seafood needs to be closely monitored, levels of serum specific IgE to tropomyosins Der p 10 and Pen a 1 may be useful biomarkers[113].

From another point of view, there is a significant prevalence of occupational asthma in shellfish-processing workers, and airborne shellfish exposure not only can cause symptoms in highly allergic subjects, but can also cause *de novo* sensitization. Symptoms may be limited to respiratory tract or may systemic, as in anaphylaxis[104,137]. Moreover, respiratory allergy to aquarium fish food (aquarium syndrome) is due to exposure to aeroallergens (either at work or as a hobby) from a variety of dried arthropod species, including common water fleas (*Daphnia* spp, crustaceans of the *Daphniidae* family), freshwater shrimps (*Gammarus* spp, crustaceans of the *Gammaridae* family), red midge larvae (*Chironomus thummi*, insect of the *Chironomidae* family), mosquito black larvae (*Culex* spp, insect of the *Culicidae* family), and segmented earthworms (*Tubifex tubifex*, annelid of the *Naididae* family). Variable degree of cross-reactivity between these arthropods with house dust mites, cockroaches and edible shrimps, was reported[137,138].

Oral mite anaphylaxis is a new syndrome characterized by severe allergic symptoms occurring immediately after eating mite-contaminated foods, in patients with a previous history of house dust mite-allergic rhinitis and/or asthma. This type of food allergy to mite ingestion was reported in various countries, including the United States, Japan, Taiwan, Venezuela, Brazil and in Southern Europe, being more prevalent in tropical/subtropical environments. Different mite species involved are house dust mites (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*), and storage mites (*Tyrophagus putrescentiae*, *Lepidoglyphus destructor*, *Blomia tropicalis etc.*). Allergenic cross-reactivity between different domestic and storage mite species could explain why patients sensitized to house dust mites may present systemic reactions when exposed to storage mites by oral route. Mite-contaminated foods are usually prepared with wheat and/or corn flour, including pancakes, sponge cakes, pizza, pasta, bread, white sauce, beignets, cornmeal cakes, and polenta. Because exposure to low temperatures inhibits mite proliferation, it is recommended to store the flour in sealed containers in the refrigerator. Other foods that can be contaminated with mites when stored for long periods at ambient temperature are cheese, ham, chorizo, and salami[139]. In Japan, cases involving ingested okonomiyaki or takoyaki prepared at home were reported, due to mite-contaminated flavored flour, the okonomiyaki-mix or takoyaki-mix being previously opened and stored for months at room temperature[140]. It is suggested that thermoresistant mite allergens are involved in pathogenesis, because many cooked mite-contaminated foodstuffs are able to induce symptoms. A variety of this syndrome is the mite ingestion-associated exercise-induced anaphylaxis[141]. Patients with oral mite anaphylaxis present also an increased prevalence of nonsteroidal anti-inflammatory drugs (NSAIDs) hypersensitivity. Even no salicylates were detected in mite-contaminated wheat flour, the opisthonotal gland secretion from *pyroglyphid* mites contains salicylaldehyde analog 2-formyl-3-hydrobenzyl formate[142]. Moreover, intake of NSAIDs sometimes enhance immediate reactions in food-dependent exercise-induced anaphylaxis[143], and salicylate hypersensitivity with reactions to salicylate food additives may occur in patients with cross-reactive NSAIDs hypersensitivity[144].

Domestic cockroaches, especially *Blattella germanica* (German cockroach), are the most important urban indoor inhalant insect allergen sources. Major German cockroach allergens, Bla g 1 and Bla g 2, are cross-reactive with similar American cockroach *Periplaneta americana* allergen components Per a 1 and Per a 2, respectively[145]. Molecular mimicry between cockroach Bla g 5 and helminth glutathione S-transferases promotes cross-reactivity and cross-sensitization[146]. Cockroaches also contain cross-reactive tropomyosin (Bla g 7), which indicates a risk for allergic reactions to shellfish or snail, which can be severe[12,103]. German cockroach allergen molecule Bla g 7 has cross-reactivity with tropomyosins from other cockroaches, such as American cockroach *Periplaneta americana* (Per a 7), but also from dust mites *Dermatophagoides pteronyssinus* (Der p 10), *Dermatophagoides farinae* (Der f 10), and ascarid nematodes *Anisakis simplex* (Ani s 3), *Ascaris lumbricoides* (Asc l 3)[22,147]. Recombinant Bla g 7 sensitization rate in German cockroach-allergic Korean patients is 16.2%[148]. Besides tropomyosin (Bla g 7), myosin light chain (Bla g 8) and arginine kinase (Bla g 9), hemocyanin is another cockroach aeroallergen (Bla g 3) cross-reactive with shellfish allergens, such as the one identified in giant keyhole limpet *Megathura crenulata* (Meg c Hemocyanin)[120,149,150].

An association between sensitization to arthropod aeroallergens and food allergy to edible insects is also possibile. The silkworm *Bombyx mori* is an important insect in the textile industry and its pupa are used in Chinese cuisine, being the most commonly eaten insect in China. The silk, urine and dander of silkworms are often the cause of allergies in sericulture workers, and silkworm pupa is known to be allergenic. Silkworm moth-sensitized patients with rhinoconjunctivitis and asthma from Southern China are frequently concomitantly sensitized to other aeroallergens from relevant mites and cockroaches (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Blomia tropicalis*, *Blattella germanica*, *Periplaneta americana*). The silk moth allergen component Bomb m 1 is the significant cross-reactive arginine kinase, similar to house dust mites and cockroaches allergenic molecules, the tropomyosin Bomb b 7 being probable less important[151-153].

Caterpillars are commonly eaten insects in Africa, ingested Mopane worm *Imbrasia belina* being reported as a cause of allergic anaphylaxis in a Zimbabwean adolescent with IgE sensitization to house dust mites and cockroaches (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Blattella germanica*), suggesting cross-reactivity due to glutathione transferases (Der p 8, Der f 8, Bla g 5) or tropomyosin[154].

Infestation of food with insects is a different type of allergy described in Spain, lentil pest *Bruchus lentis* proteins being a cause of IgE-mediated rhinoconjunctivitis and asthma in patients eating or inhaling infested legume particles[155].

Cochineal red/carmine is a natural red color, used as food additive (E120, FDA 73.100) or pharmaceutical excipient, and obtained from the dried bodies of the female scale insect *Dactylopius coccus*, which contain dye protein residues attributed to IgE-mediated sensitization, food allergy, including anaphylaxis, and occupational asthma and rhinoconjunctivitis. Insect-derived proteins possibly complexed with carminic acid may be responsible for carmine allergy, and a 38 kDa major allergen in cochineal extract was described as an insect phospholipase or related enzyme. Carmine insect allergens can act both *via* inhalation and digestion, inducing both respiratory allergy and alimentary allergy[156-159].

Aeroallergens and food allergens of vertebrate animal origin

Syndromes and associations related to clinical cross-reactivity bewteen aeroallergens and food allergens of mammalian and avian origin are described below.

Domestic mammals with fur kept as pets induce respiratory symptoms in allergic patients. The popularity of cats and dogs as pets put them among the most important sources of indoor allergens. In Europe and United States of America, at least one person in four is exposed every day to aeroallergens of mammalian origin, and almost everyone is occasionally exposed to inhalant allergens from pets or domesticated animals[160]. Specific *Carnivora* order pet allergen components are described for cat (*Felis catus* syn. *Felis domesticus*): Fel d 1, Fel d 4, and dog (*Canis lupus familiaris* syn. *Canis familiaris*): Can f 1, Can f 2, Can f 5[18,161,162].

Cross-reactive serum albumins (66-69 kDa) from mammals kept as pets or domestic animals are described as allergen components: cat *Felis domesticus* Fel d 2, dog *Canis domesticus* Can f 3, horse *Equus caballus* Equ c 3, cattle *Bos domesticus* Bos d 6, pork *Sus scrofa domestica* Sus s 6, rabbit *Oryctolagus cuniculus* Ory c 6. Allergic sensitization to serum albumins can occur by inhalation as well as ingestion. These proteins are a major component in the circulatory system of mammals, contributing to colloid osmotic blood pressure and the transport of many ligands. As important allergen components, they are present in body fluids, including saliva, in meat, and on dander. IgE cross-reactivity between inhaled (aeroallergens from pets or occupational settings) and ingested or systemically administered serum albumins must be considered in clinical practice[160,163-165].

The cat-pork syndrome consists primarily of IgE-mediated respiratory symptoms following exposure to cat dander, and secondarily of food allergy symptoms after the ingestion of pork meat[166]. The first report on cat-allergic patients experiencing anaphylaxis to pork meat suggested cross-reactivity due to a 67 kDa protein[167], later on the sensitization to cat serum albumin being considered an useful biomarker of possible cross-sensitization not only to porcine serum albumin, but also to other mammalian serum albumins[168]. Despite being a dominant protein in dander, Fel d 2 is a 67-kDa serum albumin regarded as a minor cat

allergen, about 15%-35% of cat allergic patients being sensitized to it. Only 1%-3% of cat-allergic patients seem to be at risk for food allergy to pork meat, under the circumstances that about a third of the subjects sensitized to porcine serum albumin are likely to present allergic reactions to pork consumption[160,165,168].

Although the term cat-pork syndrome seems to be appropriate because the sensitization to cat serum albumin represents the primary event in this cross-reactivity entity, it is also frequently named pork-cat syndrome. Clinical picture varies from oral itching to anaphylaxis. Because albumin is a heat-labile protein, fresh meat or dried and smoked pork are more consistent triggers than well-cooked meat. Pork grilled meat, sausages, ham and pork ribs, hamburger or barbecue were mentioned as causative factors. Small amount of pork meat in a strip of bacon or cooked pork meat may be tolerated without severe reactions, as well as seasoned pork products, such as salami. Fatal anaphylaxis after eating wild boar meat was reported in a patient with pork-cat syndrome. Symptoms usually occur within 30-45 min after eating pork meat, and does not appear to be related to tick bites. In general, patients with pork-cat syndrome, neither react to beef, nor have serum evidence of sensitization. These aspects are helpful in differentiating from delayed food allergy to red meat, due to IgE antibodies to alpha-gal (galactose-alpha-1,3-galactose, a nonprimate mammalian oligosaccharide epitope), in patients with recent tick bite/bites (1-4 wk). These alpha-gal allergic patients present delayed anaphylaxis, angioedema or urticaria, 3-6 h after eating red meat (beef, pork, lamb), but not chicken, turkey, or fish. Both cross-reactivity syndromes do not appear early in life, most reported patients are older than age of five years, with the majority being adults or adolescents[168-171].

Pig allergy was reported from the point of view of food sensitization, but also as occupational allergy. An unusual case of occupational asthma resulting from pork-cat syndrome was also recently described in a female patient having respiratory allergy with sensitization to cat dander, working at a grocery store selling cured meats (having the duty to cut pork bones), and presenting symptoms caused by inhalation. In this case cat dander was the primary sensitizer and sensitization to galactose-α-1,3-galactose, a source of cross-reactivity between meat and dander was ruled out[172]. Pig hair and dander are also important inducers of occupational allergies in farmers exposed in swine production. Moreover, popular uncommon pets include small pigs, mini-pigs, or teapot pigs[163].

Several other cross-reactivity associations between mammalian inhalant allergies with subsequent food allergy were also reported. A case of confirmed occupational respiratory allergy due to pork was followed by food allergy to pork, and later by food allergy to chicken. Porcine and chicken hemoglobin were found to be cross-reactive allergens. Although IgE cross-reactivity is most frequent between mammalian albumins, cross-reactions may also occur between cat and chicken albumins, which share 46% identical amino acids. Cross-reactivity between porcine and chicken serum albumins was possibly linked to a prior sensitization to cat serum albumin[173]. A case of occupational asthma induced by the inhalation of bovine serum albumin powder in a laboratory researcher, was followed by symptoms of food allergy after drinking milk, without symptoms on ingesting beef, pork, or chicken meat[174]. It is important to mention that milk allergic children sensitized to the allergen component Bos d 6 (bovine serum albumin) may also have concomitant beef allergy[175]. Another patient diagnosed with initial sensitization to inhaled rabbit products (such as epithelium, urine, serum) in childhood presented anaphylaxis with severe bronchospasm secondary to ingestion of rabbit meat in adolescence, the allergen involved being the 60-kDa albumin, responsible for cross-reactivity between rabbit epithelium and rabbit meat[176].

Airborne exposure to pet birds antigens may cause allergic rhinitis and/or asthma in IgE sensitized patients or hypersensitivity pneumonitis/extrinsic allergic alveolitis in others.

The bird-egg syndrome consists of primary IgE-mediated sensitization with respiratory symptoms to exposure to bird aeroallergens, and secondarily of allergy symptoms after the ingestion of eggs. This syndrome is due to cross-reactivity between egg yolk and bird allergens (feathers, serum, droppings, and meat). Its pathomechanism is different from hypersensitivity pneumonitis induced by bird antigens, such as pigeon fancier's lung. There are also differences between the bird-egg syndrome and the common allergy without sensitization to bird proteins. Patients with bird-egg syndrome are typical adults, with allergic rhinoconjunctivitis and/or asthma due to repeated exposure to household pet birds, such as budgerigars, canaries, parrots or lovebirds, and the symptoms associated with egg ingestion are usually gastrointestinal, but also cutaneous or respiratory. Food-dependent, exercise-induced anaphylaxis to egg has also been reported. While ovomucoid (Gal d 1), ovalbumin (Gal d 2), ovotransferrin (Gal d 3) and lysozyme (Gal d 4) are involved in common hen's egg white allergy, alpha-livetin found in egg yolk also known as chicken serum albumin (Gal d 5) is the allergen component involved in both respiratory and food-allergy symptoms in the bird-egg syndrome. Gal d 5 is a water-soluble, partially heat-labile, 70 kDa allergen present in egg yolk and avian meat and serum, and induce cross-reactivity to bird allergens, egg yolk, and chicken meat. A minority of patients with egg allergy are reactive to chicken meat. The role in food allergy of several other allergens identified in egg yolk, including apovitellenin I (Gal d Apo I) and apovitellenin VI (Gal d Apo VI), is still unclear[163,177-180]. Two similar cross-reactivity syndromes must also be mentioned. While the bird-egg syndrome is described in patients primarily sensitized to bird antigens, the egg-bird syndrome was reported

in patients in which egg allergy started in infancy and the primary sensitization was to egg yolk. The egg-egg syndrome is an occupational respiratory allergy to airborne egg proteins with subsequent nutritive egg allergy, in bakery and confectionery industry workers[181].

CONCLUSION

The knowledge of significant syndromes and associations related to cross-reactive allergen components and the impact of relevant cross-reactivities between aeroallergens and food allergens are of great importance for the allergy specialist. Allergen cross-reactivity may be an underestimated problem in clinical practice[13,182]. Moreover, molecular-based allergy diagnosis is essential for an accurate allergy evaluation of cross-reactions, sometimes with impact on the therapeutic strategy[12]. Patients allergic to certain food allergens and inhaled allergens should be carefully instructed about cross-reactions to other food allergens[183]. Dietary avoidance of foods that are related and have potentially cross-reactive proteins should be individualized according to the risk of clinical cross-reactivity[3].

Very recently, a trustable expert system was developed to support the interpretation of molecular tests for allergy based on microarrays. Allergen microarrays facilitate the simultaneous testing of more than 100 allergen components, represent the state-of-the-art technology for allergy diagnosis in poly-sensitized patients, and have an important role in the accurate diagnosis of syndromes and associations related to the IgE sensitization to cross-reactive allergens components. A section termed "post molecular anamnesis" suggests any clinical supplemental questions that should arise from the microarray interpretation[11,184-186].

Because the era of the characterization of molecular features of food allergens has begun, new data started to bring useful information about cross-reactivity between different sources of food allergens and aeroallergens in order to help the clinicians to provide appropriate prophylaxis approach, and to estimate the types and severity of allergic reactions[187].

Component-resolved diagnosis is a research method that explains on molecular level allergen cross-reactivity, and allows to distinguish cross-reactions occurring after ingestion of food in patients with IgE sensitization primarily to aeroallergens from the coexistence of inhaled and food allergies. Due to the geographic diversity resulting in different exposure to airborne allergens and dietary factors, studies on allergen components in populations living in different climatic zones give different results. This suggests that the diagnostic and prognostic assessment based on the component-resolved diagnosis results is limited and should always be considered in clinical context[188].

Medical history and diagnosis approach may be guided by the knowledge about the diverse cross-reacting allergens involved, and by the understanding of these clinical entities which may vary significantly or may be overlapping. The use of molecular-based allergy diagnosis improves the understanding of clinically relevant cross-reactive allergen components from aero-allergen sources and foods.

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EDITORIAL

Exercise for tendinopathy

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Abstract

Tendinopathies are one of the most common sports/musculoskeletal injury in modern western societies. Many physiotherapy approaches have been recommended in the literature for the management of tendinopathy. The most effective treatment in the management of tendinopathy is the eccentric training. Load, speed and frequency of contractions are the three principles of eccentric exercises, discussed in this report. However, eccentric training is not effective for all patients with tendinopathy and the effectiveness of this approach when applied as monotherapy is lower than it is applied as part of the rehabilitation process. For this reason, clinicians combine eccentric training with other physiotherapy techniques such as stretching, isometric and lumbar stability exercises, electrotherapy, manual therapy, soft tissue manipulation techniques, taping and acupuncture in the management of tendinopathies. Further research is needed to find out which treatment strategy combined with eccentric training will provide the best results in the rehabilitation of tendinopathy.

Key words: Tendinopathy; Exercise; Physiotherapy; Electrotherapy; Eccentric exercises; Stretching exercises; Electrotherapy; Manual therapy

Core tip: Eccentric exercises are effective in the management of tendinopathy. Eccentric training improves patients' symptoms and reverses tendinopathys' pathology. The ideal eccentric protocol is unknown in the literature. Eccentric training alone does not respond positively in many patients. Therefore, clinicians combine eccentric training with other forms of therapy such as stretching exercises, isometric contraction, electrotherapy, manual therapy, deep transverse friction, taping, acupuncture and improvement of lumbo - pelvic control. More research is needed to find out which treatment strategy combined with eccentric training will provide the best results in the rehabilitation of tendinopathy.

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Tendinopathies are one of the most common sports/musculoskeletal injury in modern western societies.

Table 1 Recommended therapies for the management of tendinopathy

Exercise	Electrotherapy	Manual therapy	Other therapies
Eccentric	Therapeutic ultrasound	MWMT for LET	DTFM
Stretching	Laser	Cyriax for LET	Taping
Isometric	ESWT		Acupuncture
Lumbo - pelvic control for lower limb tendinopathies	Iontophoresis		

ESWT: Extracorporeal shockwave therapy; LET: Lateral elbow tendinopathy; DTFM: Deep transverse friction massage; MWMT: Mobilization with manual therapy.

The most common tendinopathies of the upper limb are Rotator cuff (mainly supraspinatus) and lateral elbow tendinopathy (LET) usually seen in sports such as volley - ball, tennis, basketball, swimming and so on whereas Achilles and patellar tendinopathy are the most common tendinopathies of the lower limb commonly seen in sports such as volley-ball, soccer, running, jumping and so on[1]. Many physical therapy strategies have been proposed for the rehabilitation of tendon disorders. These strategies are electrotherapeutic such as therapeutic ultrasound, extracorporeal shockwave therapy, low level laser, iontophoresis and non-electro-therapeutic modalities such as eccentric training, soft tissue techniques, taping and needle therapy (Table 1). These treatments intend to reduce pain and improve function in tendinopathy but act in a totally different mechanism of action. Generally speaking, the efficacy of a treatment is based on reversing the pathology of the tendinopathy and not only improving the symptoms. Nowadays, eccentric exercise program is the most effective conservative approach in the treatment of tendinopathy[2,3].

Load, speed and frequency of contractions are the three principles of eccentric exercises. The results are poor when the load of eccentric exercises should not be increased according to the patient's symptoms[4]. It is impossible to standardize the rate of increase of the load during the treatment period[5] but if the eccentric loading exercise can be performed without experiencing any minor pain or discomfort, it will be increased by adding weight.

The speed of eccentric training should be increased in every treatment session[6,7], Stanish *et al*[8] (2000) state to simulate the mechanism of injury, which usually occurs at relatively high velocities the load on the tendon should be increased. However, to allow tissue healing and to avoid the possibility of re-injury, eccentric exercises should be performed at a slow velocity[9]. Low velocity eccentric loading generates less injurious heat within the tendon and does not exceed the elastic limit of the tendon[10]. It is not possible to define the "slowness" of eccentric contractions. This lack of definition is based on the therapists' claim that patients perform the eccentric exercises slowly anyway in order to avoid pain[11]. However, the slowness of eccentric training should be defined when researchers develop an exercise programme treatment protocol. It is difficult for therapists to replicate the exercise training and put it into practice when the slowness is not defined.

Repetitions and sets can vary in the literature. Three sets of 15 repetitions are usually recommended. The sets are performed once or twice per day. The performance of sets based on home or supervised eccentric training. An exercise programme that can be performed any time during the day without requiring supervision by a physiotherapist called home exercise programme. The pain in patellar tendinopathy[2]. Achilles tendinopathy[2] and LET[11,12] was reduced when a home exercise program was performed for about three months. Patients fail to comply with this regimen[13,14]. The solution in the above problem is to be performed an exercise program in a clinical setting under the supervision of a physiotherapist. The supervised exercise programme may give good long-term results in one month[15-20]. This occurred because a higher degree of patient compliance can be achieved by the supervised exercise programme.

Eccentric programme reduces the pain and improves the function in all sites of tendinopathy? For example, patients with mid-portion Achilles tendinopathy respond positively in eccentric training with dorsiflexion[21-26], but patients with insertional Achilles tendinopathy respond positively in eccentric training without dorsiflexion[27]. Therefore, the two sites of Achilles tendinopathy respond positively in two different protocols of eccentric training. Patients with patellar tendinopathy at the inferior pole of the patella respond positively in squats[2]; however, the effectiveness of eccentric loading training programme on other sites of patellar tendinopathy has not been investigated. Thus, research is needed to determine the effectiveness of eccentric training at all sites of tendinopathies.

Eccentric training alone is not effective for many patients with tendinopathies[9]. Therefore, eccentric training is combined with static stretching exercises in the treatment of tendinopathies with positive results[15-20]. The way that eccentric and stretching exercises reverse the pathology of tendinopathy is unknown because evidenced - based studies to confirm that physiological effects translate into clinically meaningful outcomes and vice versa are lacking. In addition, research supports that the combination of eccentric training, with a physical therapy modality, such as therapeutic ultrasound[28,29], low level laser[30], extracorporeal shockwave therapy[31] and iontophoresis[32], is more effective therapeutic approach than the eccentric training alone

in the rehabilitation of tendinopathy. Furthermore, clinicians thought that patients with patellar and Achilles tendinopathy have lack of lumbopelvic control (lumbopelvic control defines as the reestablishment of the impairment or deficit in motor control around the neutral zone of the spinal motion segment) and this loss has the potential to alter load distribution on the lower limb kinetic chain[33]. My colleagues and I think that the improvement of lumbo-pelvic control can be achieved by performing simple exercises such as single leg bridging in supine and four point prone bridging exercises. Future research is needed to confirm the above relief. Furthermore, a plethora of manual therapies have been advocated for the management of tendinopathy, but there is minimal experimental evidence to support the efficacy of the use of manual therapy for the management of tendinopathy[34]. Mulligan Mobilization with Movement and Cyriax physiotherapy are the most common manipulative techniques for the management of LET. It is unknown whether an analogous manipulation procedure may be found for the rehabilitation of other tendinopathies comparable to that used in management of LET or may be difficult in practice of attempting such a technique at other joints[35,36]. It is believed that even if a similar technique is found for the rehabilitation of all tendinopathies, this technique will be combined with an exercise training in the treatment of tendinopathy. Finally, a recently published case trial showed that isometric contractions of the wrist extensors as a supplement to eccentric and static exercises of wrist extensors is an effective treatment approach in a patient with LET[37]. Future trials to confirm the results of the present case report in all tendinopathies are needed.

Finally, deep transverse friction massage (DTFM), taping and acupuncture have also recommended in the management of tendinopathy. DTFM is a specific type of massage applied precisely to the tendons[35]. Details about the application and mechanism of action of DTFM can be found in the article by Stasinopoulos and Johnson[35] (2007). The conducted trials do not recommend the use of DTFM in the management of tendinopathy[15,17,38]. Taping and acupuncture improve the signs of tendinopathy but it does not reverse the pathology of tendinopathy[39,40].

In conclusion, eccentric training is the most promising treatment approach in the management of tendinopathy. The optimal protocol of eccentric training is needed to investigate. The effectiveness of this approach when applied as monotherapy is lower than it is applied as part of the rehabilitation process. Further research is needed to find out which treatment strategy combined with eccentric training will provide the best results in the rehabilitation of tendinopathy.

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EDITORIAL

Bartter and Gitelman syndromes: Spectrum of clinical manifestations caused by different mutations

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Abstract

Bartter and Gitelman syndromes (BS and GS) are inherited disorders resulting in defects in renal tubular handling of sodium, potassium and chloride. Previously considered as genotypic and phenotypic heterogeneous diseases, recent evidence suggests that they constitute a spectrum of disease caused by different genetic mutations with the molecular defects of chloride reabsorption originating at different sites of the nephron in each condition. Although they share some characteristic metabolic abnormalities such as hypokalemia, metabolic alkalosis, hyperplasia of the juxtaglomerular apparatus with hyperreninemia, hyperaldosteronism, the clinical and laboratory manifestations may not always allow distinction between them. Diuretics tests, measuring the changes in urinary fractional excretion of chloride from baseline after administration of either hydrochlorothiazide or furosemide show very little change (< 2.3%) in the fractional excretion of chloride from baseline in GS when compared with BS, except when BS is associated with *KCNJ1* mutations where a good response to both diuretics exists. The diuretic test is not recommended for infants or young children with suspected BS because of a higher risk of volume depletion in such children. Clinical symptoms and biochemical markers of GS and classic form of BS (type III) may overlap and thus genetic analysis may specify the real cause of symptoms. However, although genetic analysis is available, its use remains limited because of limited availability, large gene dimensions, lack of hot-spot mutations, heavy workup time and costs involved. Furthermore, considerable overlap exists between the different genotypes and phenotypes. Although BS and GS usually have distinct presentations and are associated with specific gene mutations, there remains considerable overlap between their phenotypes and genotypes. Thus, they are better described as a spectrum of clinical manifestations caused by different gene mutations.

Key words: Gitelman syndrome; Bartter syndrome; Potassium; Chloride; Magnesium; Metabolic alkalosis; Genetics

Core tip: As inherited disorders of renal tubular excretion and reabsorption of electrolytes, Bartter and Gitelman syndromes were previously considered as genotypic and phenotypic heterogeneous diseases. Although they share some characteristic features, the clinical and laboratory manifestations may not always allow distinction between them. Different genetic mutations inducing impairment of electrolytes transport across different sites of the nephron have been reported in each condition. However, considerable overlap exists between the different genotypes and phenotypes of these two conditions that are now better described as a spectrum of clinical manifestations caused by different gene mutations.

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INTRODUCTION

Bartter syndrome (BS) and Gitelman syndrome (GS) are inherited autosomal recessive conditions resulting in defects of renal tubular excretion and reabsorption of electrolytes. A brief reminder of the physiology of renal handling of water and electrolytes homeostasis is helpful to understand these two conditions.

PHYSIOLOGY[1]

Water and electrolyte homeostasis is maintained by the kidney. To avoid significant losses of electrolytes in the urine following glomerular filtration, their reabsorption in the renal tubule is required. The distal nephron reabsorbs approximately 30% of the filtered sodium: while one quarter is reabsorbed in the thick ascending limb (TAL) of Henle's loop; the distal convoluted tubule (DCT) and the cortical collecting duct (CCD) reabsorb 10%. Dysfunction of distal tubular functions, due to either genetic or acquired causes, will result in a clinical presentation specific to the affected part of the distal nephron.

The TAL is not permeable to water and reabsorbs a large proportion of the filtered sodium chloride, which leads to interstitial hypertonicity that powers the countercurrent exchange and urinary concentration mechanisms. In case of impairment of this function, a major loss of water and sodium occur, as seen with loop diuretics.

The DCT, composed of an early segment (DCT1), a late portion (DCT2), and the connecting tubule leading to the CCD, finely regulates renal excretion of sodium chloride, calcium and magnesium. These segments express the different transport proteins involved in the reabsorption sodium, calcium and magnesium and their functional impairment leads to extracellular volume depletion, initially compensated for by hyperaldosteronism and resulting in increased potassium urinary losses.

The total distal nephron has a finely regulated reabsorption capacity to cope with the intake and/or extrarenal losses of salt and water. The macula densa (MD) in the distal nephron modulates renal hemodynamics and tubular reabsorption with the modulation of the renin-angiotensin Ⅱ system and intrarenal cyclooxygenase 2 activity regulating the glomerular arterial resistance.

GENERALITIES

Although BS and GS share some characteristic metabolic abnormalities such as hypokalemia, metabolic alkalosis, hyperplasia of the juxtaglomerular apparatus with hyperreninemia, hyperaldosteronism, and, sometimes hypomagnesemia[2-4], the clinical and laboratory manifestations may not always allow distinction between them[5]. They were previously considered as genotypic and phenotypic heterogeneous diseases, with urinary calcium and prostaglandin E excretion as well as serum magnesium levels enabling distinction between them. However recent evidence suggests that they constitute, instead, a spectrum of disease characterized by defective chloride reabsorption caused by different genetic mutations at different sites of the nephron in each condition, resulting in three major types of tubulopathy[1,6]: (1) abnormality in the sodium-potassium-chloride cotransporter NKCC2 or the renal outer medullary potassium (ROMK) channel will lead to an impairment in the thick ascending limb of Henle which has a greater salt reabsorption capacity, resulting in severe polyuric loop dysfunction with major urinary salt and water losses. This condition is also known as antenatal BS or hyperprostaglandin E syndrome; (2) defects in the sodium-chloride cotransporter NCCT or the chloride channel ClC-Kb in the DCT which modulates urinary calcium and magnesium excretion, will induce hypokalemia (in BS) with hypomagnesemia (in GS); and (3) abnormality in the chloride channels ClC-Ka and ClC-Kb or their beta-subunit Barttin in combined loop and distal convoluted tubule will lead to more manifestations (antenatal BS or hyperprostaglandin E syndrome with sensorineural deafness).

CHARACTERISTICS SHARED BY BOTH CONDITIONS

Both conditions are inherited in an autosomal recessive mode. Chronic hypokalemia results in fatigue, dizziness, constipation, muscle cramps and weakness. Although usually mildly symptomatic, hypokalemia can be ex-

Table 1 Genetics and presentation of Bartter and Gitelman syndromes

Disorder	Gene affected	Gene product	Clinical presentation
Bartter syndrome type I	SLC12A1	NKCC2	Antenatal Bartter syndrome (hyperprostaglandin E syndrome)
Bartter syndrome type II	KCNJ1	ROMK	Antenatal Bartter syndrome
Bartter syndrome type III	ClC-Kb	CLC-Kb	Hypochloremia, mild hypomagnesemia, failure to thrive in infancy
Bartter syndrome type IVA	BSND	Barttin (B-subunit of CLC-Ka and CLC-Kb)	Antenatal Bartter syndrome (hyperprostaglandin E syndrome) and sensorineural deafness
Bartter syndrome type IVB	ClC-Ka and ClC-Kb	CLC-Ka and CLC-Kb	Antenatal Bartter syndrome (hyperprostaglandin E syndrome) and sensorineural deafness
Bartter syndrome type V	*CaSR* gene	CaSR	Bartter syndrome with hypocalcemia
Gitelman syndrome	SLC12A3	NCC	Hypomagnesemia, hypocalcuria, growth retardation

There are six Bartter syndrome subtypes (I, II, III, IV, IVB, and V) corresponding to six genetic defects. Modified from Seyberth *et al*[6]. NKCC2: Furosemide-sensitive sodium-potassium-2 chloride cotransporter; ROMK: Renal outer medullary potassium channel; CLC-Kb: Chloride channel Kb; CLC-Ka: Chloride channel Ka; CaSR: Calcium sensing receptor; NCC: Thiazide-sensitive sodium-chloride cotransporter.

acerbated by fluid and electrolytes losses caused by diarrhea or vomiting, or by abuse of alcohol, cocaine or other drugs, and can lead to rhabdomyolysis, prolonged QT interval, life-threatening arrhythmia, syncope and sudden death[7,8].

Biochemical findings common to both conditions include hypokalemia, hypochloremia and metabolic alkalosis associated with hyperreninemia and hyperaldosteronism. Hypomagnesemia used to be considered a feature of GS; however, many reports have also described it in patients with BS[9,10].

DISTINCTIVE CHARACTERISTICS OF BS

The defect of NaCl reabsorption in the thick ascending limb of Henle's loop is central to the pathophysiology of BS[3]. The condition has a prevalence of approximately 1.2 per million[11]. Severe failure to thrive commonly presents in early childhood. Blood pressure is usually normal. BS is classified into five subtypes corresponding to specific defective transport proteins in the renal tubules secondary to different gene mutations[12] as shown in Table 1.

At the luminal (urinary) side of the terminal ascending loop

BS type Ⅰ or antenatal Bartter syndrome or hyperprostaglandin E syndrome. This autosomal recessive condition is caused by mutations of the *SLC12A1* gene coding the Na-K-Cl co-transporter protein in the renal tubule. Antenatal manifestations such as polyhydramnios secondary to fetal polyuria may occur. As transepithelial voltage gradient cannot be maintained to absorb calcium and magnesium, hypercalciuria and hypermagnesiuria occur and may result in nephrocalcinosis. There is no associated sensorineural deafness.

BS type Ⅱ or neonatal Bartter syndrome with transient hyperkalemic metabolic acidosis or antenatal Bartter syndrome. This autosomal recessive condition is caused by mutations of the *KCNJ1* gene that codes the inward rectifying ROMK channel. The initial neonatal presentation is hyperkalemic, metabolic acidosis that may mimic pseudohypoaldosteronism. Antenatal manifestations such as polyhydramnios secondary to fetal polyuria may occur. There is no associated sensorineural deafness.

On the basal lateral (blood) side of the terminal ascending loop

The autosomal recessive BS type Ⅲ or classic BS is caused by a defect in the chloride channel Kb (ClC-Kb) secondary to mutations encoding the basolateral chloride channel. As ClC-Ka chloride permeability is preserved, the symptoms are usually very mild but might overlap with those of GS because the ClC-Ka is also present in the DCT. Sensorineural deafness, nephrocalcinosis and nephrolithiasis do not occur.

The autosomal recessive BS type Ⅳ or antenatal BS with sensorineural deafness is due to *BSND* gene mutations leading to an altered Barttin β-subunit of both chloride channel Ka (ClC-Ka) and ClC-Kb needed for potassium chloride membrane localization. As both ClC-Ka and ClC-Kb channels are affected, the symptoms are usually severe and may initially mimic pseudohypoaldosteronism. However, when the ROMK channel and other potassium channels or transporters start to compensate, the neonates develop hypokalemia and metabolic alkalosis. The resulting disturbances in potassium transport cause the sensorineural deafness, because cochlear hearing function relies on several processes governing potassium flow such as its inflow into cochlear hair cells, its entry into hair cells by electro-chemical forces and its recycling either *via* KCNQ4 channels or by entering Deiter's cells (*via* KCC3, KCC4)[13-15].

BS type V is an autosomal dominant condition caused by *L125P* mutations of the extracellular basolateral calcium sensing receptor located on chromosome 16q13. This results in hypocalcemia hypercalciuria and suppression of parathyroid hormone function, asso-

Table 2 Features differentiating Bartter and Gitelman syndromes

Features	Classic Bartter syndrome	Gitelman syndrome
Age at onset	Childhood (early)	Childhood or later
Maternal hydramnios	Rare	Absent
Polyuria, polydipsia	Present	Rare
Dehydration	Often present	Absent
Tetany	Rare	Present
Growth retardation	Present	Absent
Urinary calcium	Normal or high	Low
Nephrocalcinosis	Rare	Absent
Serum magnesium	Occasionally low	Low
Urine prostaglandins (PGE2)	High or normal	Normal

Modified from Urbanová *et al*[20].

ciated with Bartter-like syndrome[16,17].

DISTINCTIVE CHARACTERISTICS OF GS

The autosomal recessive GS, or familial hypokalemic metabolic alkalosis with hypomagnesemia and low urinary calcium excretion has a prevalence of approximately 1 in 40000[18]. It results from transport defects located in the DCT caused by mutations in the solute carrier family 12, member 3 gene (*SLC12A3*) that encodes the thiazide-sensitive NaCl cotransporter (NCC). Mutations in the gene encoding the chloride channel ClC-Kb have also been identified in some individuals[19].

GS is very often asymptomatic. If symptoms occur, this is usually after the age of six years but the condition is often diagnosed in adolescents or adults. The initial presentation is usually the incidental discovery of an asymptomatic and isolated hypokalemia. Some patients present with fatigue, dizziness, muscle weakness, cramps, vomiting, abdominal pain, fever, nocturia and polyuria. Facial Paresthesias may also occur and, occasionally, hypotension. Failure to thrive is not usually severe unless severe hypokalemia and hypomagnesemia are present. Hypocalciuria is a distinct feature and interstitial nephritis may develop because of the persistent hypokalemia. Adults can present with chondrocalcinosis with swollen and warm joints with overlying tenderness. Sudden cardiac arrest has been reported occasionally[8]. Hearing defect is absent. The most important differential diagnosis is BS (especially type Ⅲ). Antenatal diagnosis is available but not usually required because most patients have a good prognosis[18].

DISTINGUISHING BETWEEN THE TWO CONDITIONS

Clinical and biochemical findings

The main differences in the clinical presentation of BS and GS are explained in Table 2[20]. Although the symptoms of BS type Ⅲ (classical BS) often occur before the age of two, patients can present at any age until adolescence, with an initial history of polyuria and polydipsia, followed by growth retardation if the diagnosis and treatment were delayed[21]. Patients usually have high urinary prostaglandins E2 (PGE2) production and hypercalciuria[1,22]. However, the distinction between BS and GS is not always that simple because phenotypic variances. Although genetic diagnosis is possible, its use remains limited because it is costly and not always readily available. In addition, its usefulness remains limited because the "hot spot" mutations along the gene are not always present.

Diuretics test: Response to thiazide and furosemide

The diuretic test involves measuring the change in the urinary fractional excretion of chloride after administration of a diuretic. This consists of either oral hydrochlorothiazide (1 mg/kg up to 50 mg) or furosemide (a single dose of 2 mg/kg). The diuretic is administered after a 7-d "washout" period, during which therapies other than potassium and magnesium supplements are withheld. In GS caused by a defect is in the thiazide-sensitive NCCT, the thiazide test results in only a minimal change (< 2.3%) in the fractional excretion of chloride from baseline. In BS with ClC-Kb mutations, this blunted response does not occur but a normal response to furosemide exists. In BS with KCNJ1 mutations, there is a good response to both diuretics[23]. Because of a higher risk of volume depletion in infants or young children, diuretic tests are not recommended for them when they are suspected to suffer from BS. In patients with the normotensive hypokalemic alkalosis phenotype, an abnormal hydrochlorothiazide test allows to predict with a very high sensitivity and specificity the GS genotype and thus avoid the need for genotyping[24].

Genetic investigations

Clinical symptoms and biochemical markers of GS and classic form of BS (type Ⅲ) may overlap and thus genetic analysis is required to make an accurate diagnosis[1]. Although genetic tests are available, they still face technical difficulties caused by large gene dimensions and the absence of hot-spot mutations. They are also lengthy and costly. Furthermore, considerable overlap exists between the different genotypes and phenotypes.

In most patients with GS, DNA variants are found in the thiazide-sensitive NaCl co-transporter (NCC) encoding *SLC12A3* gene. In others, variants in the chloride channel ClC-Kb encoding *ClC-Kb* gene are identified, causing not only classical BS (type Ⅲ), but also other phenotypes that overlap with antenatal BS (Types Ⅰ-Ⅱ) or with GS[1,3,5,12,20,25-41]. Other genetic and/or environmental factors also act as effect modifiers in other cases of ClC-Kb mutation[29,30] and in polycystic kidney disease[31]. Furthermore, in one family sharing *ClC-Kb* variant some relatives presented clinical characteristics specific for GS, on the one side of the

spectrum, to classic BS on the other[33]. As a result, screening for the *ClC-Kb* gene in patients with the GS phenotype who do not have variants in the *SLC12A3* gene is therefore required[10].

TREATMENT

Bartter syndrome

Hypokalemia, often in the range of 2-3 mmol/L, is caused by increasing urinary potassium losses due to the activation of the renin-angiotensin-aldosterone system and hyperaldosteronism secondary to salt and water depletion caused by the inability to reabsorb sodium in the TAL of the loop of Henle or the DCT. Correcting it is the mainstay of treatment.

Potassium chloride supplements are preferred salt because of the coexisting chloride deficiencies in these patients. Several hundred mmol of potassium per day may be required to correct the hypokalemia.

Spironolactone, a specific aldosterone antagonist, binds competitively binding to the receptors present at the aldosterone-dependent sodium-potassium exchange site in the DCT. It increases water excretion while retaining potassium.

By inhibiting sodium reabsorption at the DCT, cortical collecting tubule, and collecting duct, Amiloride reduces potassium and hydrogen excretion.

By interfering with the active transport exchange of potassium and sodium in the distal tubule, cortical collecting tubule, and collecting duct Triamterene decreases calcium excretion and increases magnesium loss.

Angiotensin-converting enzyme (ACE) inhibitors, such as captopril, enalapril and lisinopril, block the conversion of angiotensin Ⅰ (ANG Ⅰ) to ANG Ⅱ and prevent the secretion of aldosterone from the adrenal cortex.

Nonsteroidal drug anti-inflammatory drugs (NSAID) decrease prostaglandin PGE2 synthesis, which causes the pressor resistance to ANGⅡ and norepinephrine, hyperreninemia, and increased sympathoadrenal activity. The resulting hyporeninemic hypoaldosteronism leads to potassium retention. Medications include indomethacin and naproxen which decrease the activity of the enzyme cyclo-oxygenase (COX) which increases prostaglandin synthesis.

Administration of growth hormone (GH) is required for the treatment of short stature and growth failure, which are common.

In the presence of muscle spasms or tetany, calcium or magnesium supplements may be required.

GS

Asymptomatic patients often require no treatment but need outpatient monitoring once or twice yearly. A high-sodium and potassium diet is recommended.

Lifelong magnesium supplementation is required. As high doses of magnesium cause diarrhea, normalization of serum magnesium level is difficult to achieve. Oral magnesium-chloride supplementation is initially started with a daily dose of 3 mmol/m^2 or 4-5 mg/kg, divided in 3-4 administrations to avoid diarrhea. The dose will subsequently be adjusted according to serum magnesium levels. It has also to be increased during periods of intercurrent illness, especially in the presence of vomiting and diarrhea. If tetany develops, intravenous administration of 20% $MgCl_2$ (0.1 mmol mg/kg per dose) should be administered and can be repeated every 6 h if needed.

Hypokalemia may require large amounts of potassium chloride supplements, up to 10 mmol/kg in children and 500 mmol/d in adults, but poor gastric tolerance frequently occurs. If symptomatic, hypokalemia is treated by a combination therapy of amiloride (5–10 mg/1.73 m^2 per day) and spironolactone (200-300 mg/d), in addition to KCl supplementation (1-3 mmol/kg per day divided in 3-4 doses). Amiloride therapy should be started with a lower dose initially to avoid the development of hypotension.

Symptomatic chondrocalcinosis (pseudo-gout attacks) requires NSAID.

PROGNOSIS

Bartter syndrome

The prognosis depends on the degree of the receptor dysfunction. Without treatment, there is significant morbidity and mortality. Once treated, most patients lead fairly normal lives. Nearly all patients have growth retardation and/or short stature, which improve with potassium, indomethacin, and GH therapy. A small proportion of patients develops slow progression to chronic renal failure, due to interstitial fibrosis, and may require renal replacement therapy. Nephrocalcinosis may occur and is often associated with hypercalciuria. Cardiac arrhythmias, sometimes leading to sudden death, may occur when significant electrolyte imbalances are present. Sensorineural deafness, associated with Bartter syndrome IV, requires appropriate treatment.

GS

The long-term prognosis is generally excellent. The musculoskeletal and constitutional symptoms, the nocturia and polydipsia, may seriously hamper the daily activities and negatively affect the patients' quality of life. There is a risk of developing sudden cardiac arrhythmias, sometimes life-threatening, especially in the presence of severe hypokalemia, hypomagnesemia and alkalosis. These episodes are sometimes precipitated by non-adherence to therapy, the presence of concomitant diarrhea or vomiting or competitive sports that induce potassium and magnesium loss by sweating.

CONCLUSION

Although BS and GS usually have distinct presentations and are associated with specific gene mutations, there remains considerable overlap between their phenotypes

and genotypes. Thus, they are better described as a spectrum of clinical manifestations caused by different gene mutations.

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EDITORIAL

Neurally adjusted ventilator assist in very low birth weight infants: Current status

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Abstract

Continuous improvements in perinatal care have resulted in increased survival of premature infants. Their immature lungs are prone to injury with mechanical ventilation and this may develop into chronic lung disease (CLD) or bronchopulmonary dysplasia. Strategies to minimize the risk of lung injury have been developed and include improved antenatal management (education, regionalization, steroids, and antibiotics), exogenous surfactant administration and reduction of barotrauma by using exclusive or early noninvasive ventilatory support. The most frequently used mode of assisted ventilation is pressure support ventilation that may lead to patient-ventilator asynchrony that is associated with poor outcome. Ventilator-induced diaphragmatic dysfunction or disuse atrophy of diaphragm fibers may also occur. This has led to the development of new ventilation modes including neurally adjusted ventilatory assist (NAVA). This ventilation mode is controlled by electrodes embedded within a nasogastric catheter which detect the electrical diaphragmatic activity (Edi) and transmit it to trigger the ventilator in synchrony with the patient's own respiratory efforts. This permits the patient to control peak inspiratory pressure, mean airway pressure and tidal volume. Back up pressure control (PC) is provided when there is no Edi signal and no pneumatic trigger. Compared with standard conventional ventilation, NAVA improves blood gas regulation with lower peak inspiratory pressure and oxygen requirements in preterm infants. NAVA is safe mode of ventilation. The majority of studies have shown no significant adverse events in neonates ventilated with NAVA nor a difference in the rate of intraventricular hemorrhage, pneumothorax, or necrotizing enterocolitis when compared to conventional ventilation. Future large size randomized controlled trials should be established to compare NAVA with volume targeted and pressure controlled ventilation in newborns with mature respiratory drive. Most previous studies and trials were not sufficiently large and did not include long-term patient oriented outcomes. Multicenter, randomized, outcome trials are needed to determine whether NAVA is effective in avoiding intubation, facilitating extubation, decreasing time of ventilation, reducing the incidence of

CLD, decreasing length of stay, and improving long-term outcomes such as the duration of ventilation, length of hospital stay, rate of pneumothorax, CLD and other major complications of prematurity. In order to prevent barotrauma, next generations of NAVA equipment for neonatal use should enable automatic setting of ventilator parameters in the backup PC mode based on the values generated by NAVA. They should also include an upper limit to the inspiratory time as in conventional ventilation. The manufacturers of Edi catheters should produce smaller sizes available for extreme low birth weight infants. Newly developed ventilators should also include leak compensation and high frequency ventilation. A peripheral flow sensor is also essential to the proper delivery of all modes of conventional ventilation as well as NAVA.

Key words: Interactive ventilatory support; Positive-pressure respiration; Diaphragm; Premature; Very low birth weight; Respiratory distress syndrome; Electrical diaphragmatic activity; Synchrony; Neural triggering

Core tip: Neurally adjusted ventilator assist (NAVA) ventilation utilizes the patient's neural respiratory drive to synchronize ventilatory support on a breath-by-breath basis based on the infant's ongoing needs. It appears to work well in neonates but evidence that it makes a difference in outcomes in this population has not been established so far. The majority of studies have shown no significant adverse events in neonates ventilated with NAVA nor a difference in the rate of intraventricular hemorrhage, pneumothorax, or necrotizing enterocolitis when compared to conventional ventilation. The challenge for neonatal health care providers remains the steep and prolonged learning curve for the application of NAVA.

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INTRODUCTION

Survival of more and more premature infants has occurred as a result of continuous improvements in perinatal care. Their extremely immature lungs are prone to injury with mechanical ventilation because the gas volumes/kg body weight of the lungs are small[1]. Lung injury is inversely related to gestational age[2]. This injury may develop into chronic lung disease (CLD) or bronchopulmonary dysplasia (BPD). Strategies to minimize the risk of lung injury have been developed and include improved antenatal management (education, regionalization, steroids, and antibiotics), exogenous surfactant administration and reduction of barotrauma by using exclusive or early noninvasive ventilatory support[2,3].

The most frequently used mode of assisted ventilation is pressure support ventilation (PSV)[4-6]. Spontaneous breathing is detected by changes in airway flow or pressure in order to coordinate the ventilatory assist[7]. However, poor patient-ventilator interaction might result from conventional pneumatic triggering of the ventilator[8]. Furthermore, the potentially beneficial variability of the breathing pattern of the patient is not supported by PSV, as fixed pressure support is delivered regardless of the patients' needs[9]. This inability to provide synchrony between the patient and the assist delivered has also been demonstrated in children[10]. Patient–ventilator asynchrony has been associated with poor clinical outcome[11-15].

Changes in diaphragm structure occur following prolonged mechanical ventilation in animal models[14]. Rapid disuse atrophy of diaphragm fibers also occurs in mechanically ventilated humans[15]. This diaphragmatic muscle "disuse atrophy" "ventilator-induced diaphragmatic dysfunction" caused by sustained inactivity of the respiratory muscles (*i.e.*, passive ventilation) results in acute inflammation, loss of muscle mass, deconditioning and weakness in animal models and also in humans[6]. The preservation of spontaneous breathing during mechanical ventilation not only helps to preserve diaphragmatic function, but also to avoid atelectasis and improve oxygenation[11-15].

NEURALLY ADJUSTED VENTILATOR ASSIST

Why was neurally adjusted ventilator assist introduced and how does it work?

Over the past few years, new ventilation modes have been developed with different new strategies implemented to wean from mechanical ventilation early in order to reduce the occurrence of ventilator-induced lung injury. As patient-ventilator synchrony is essential, spontaneous patient's breathing with mechanical ventilation should be maintained whenever possible, with mechanical ventilation delivering exactly the support needed by the patient. When initiated by the patient each breath is supported immediately and that support is tailored breath-by-breath by the patient's current needs[16]. In addition, it is equally important that the level of mechanical assisted ventilation does not exceed the patient's needs. All these considerations have led to the development of neurally adjusted ventilatory assist (NAVA)[16-18]. This ventilation mode is controlled by an array of eight bipolar electrodes (sensors) electrodes embedded within a specialized nasogastric catheter positioned at the level of the crural diaphragm. These sensors detect the electrical diaphragmatic activity (Edi), and filter from electrical contamination from the heart, esophagus, and environment before

transmitting it to trigger the ventilator in synchrony with the patient's own respiratory efforts[18]. The ventilator breath is triggered and terminated by changes in this electrical activity, with the delivered inspiratory pressure proportional to the electrical signal, permitting the patient to control peak inspiratory pressure, mean airway pressure and tidal volume. In addition, as it is the patient who initiates and terminates the breaths, he also determines inspiratory (IT), expiratory times (ET) and respiratory rate, enabling flexible ventilation with breath-to-breath variability[17]. Breaths are generally initiated at 0.5 microvolt above the minimum Edi (Edi min) and terminated when the Edi signal has fallen to 70% of its peak value. The pressure curve in NAVA follows the Edi signal pattern. Back up pressure control (PC) is provided when there is no Edi signal and no pneumatic trigger. The pressure gradient (ΔP) is directly proportional to the ΔEdi following the equation (Δ P = ΔEdi × NAVA level) allowing a proportional assist mode of ventilation, which provides more support to distressed patients and permits spontaneous weaning once the lung compliance improves. NAVA levels between 1 and 4 cm H_2O/microvolt are generally used to augment respiratory support based on the size of the Edi signal aiming for normal Edi values between 5 and 10 microvolts. Therefore, if the Edi values are higher than normal, increasing ventilatory support may be considered for unloading the patient's diaphragm, while if the Edi values are lower than normal, reducing ventilatory support may be required instead in order to exercise diaphragmatic muscle fibers[18]. The tidal volume is lower than that of conventional ventilation potentially reducing lung injury and preventing disuse diaphragmatic atrophy. Failure to detect an Edi signal may result from respiratory center failure to deliver a signal (*e.g.*, apnea of prematurity, central hypoventilation syndrome, brain injury, sedation), diaphragmatic hernia, phrenic nerve conduction failure or chemical paralysis of the neuromuscular junction or the diaphragm[18]. Patients with diaphragmatic hernia are generally able to produce an Edi signal sufficient to allow assisted ventilation with NAVA[19,20].

Types of NAVA

Invasive NAVA is a complex mode of ventilation that combines NAVA, PS and PC in various proportions with short periods of apnea. Backup PC starts when the pneumatic and the neural trigger are both delayed for a period of time set by the clinician between 2 and 10 s. An automatic switch to the pressure support (PS) mode occurs in invasive NAVA modem when neural IT exceed 50% to 60% of the total respiratory time over 20 s. However, the clinician cannot set an upper limit to the IT for each breath.

Non-invasive NAVA (NIV-NAVA) is technically identical to invasive NAVA but it provides only NAVA and backup PC. Pneumatic triggers and PS are taken out of the loop because of the extremely high air leak that may reach 99% in non-invasive ventilation. Weaning occurs spontaneously but this is not the case in the backup PC mode where (PIP) is set manually. Frequent monitoring of the inspiratory pressure in NAVA should be used as a guide to select the appropriate PIP for the PC backup mode.

Advantages of NAVA

In neural triggering, the electrical trigger coming from the brain through the vagal nerve stimulates diaphragm as the same time as the ventilator, improving therefore patient-ventilator synchrony, permitting breath-to-breath variability and reducing the need for sedation[21,22]. In contrast, the pneumatic triggers used in conventional ventilation are delayed because, by definition, they occur only after the diaphragm has already contracted to generate a chest movement, a negative pressure or a positive flow. In addition neural triggering is independent of air leak around the endotracheal tube (ETT) while pneumatic triggering is sensitive to air leak. Multiple cross over trials between pressure control modes and NAVA have repeatedly shown that NAVA improves patient-ventilator synchrony in low birth weight infants, even in the presence of large air leaks[21,22]. The patient takes full control of the ventilator while receiving a timely support proportional to his own his efforts, unloading therefore the diaphragmatic muscle and reducing work of breathing[23]. This result in reduced infant fatigue and decreases the need for mandatory ventilation[16,24]. Compared with standard conventional ventilation in preterm infants, NAVA improves blood gas regulation while still using lower peak inspiratory pressure and oxygen requirements[25,26]. Edi may be used to determine optimal ventilatory support: if the infant is over-ventilated, his spontaneous respiratory drive will be suppressed resulting in a decrease of the Edi signal, while if he is under-ventilated an increased respiratory drive and higher Edi signals will result[18].

Feasibility of NAVA in very low birth weight infants

The feasibility of using NAVA in very low birth weight infants (VLBWI) was demonstrated in 2009 in a randomized crossover study on seven newborns between NAVA applied for 20 min and PSV[22]. Three other randomized crossover trials in VLBWI have compared NAVA to SIMV or PCV[23,25,27]. However they were of small sample size (n = 26, 5 and 10) and NAVA was applied for short time (4, 4 and 1 h respectively). Short-term benefits with NAVA were observed, including improved patient-ventilator synchrony and the need for a lower PIP to produce the same PaCO2. NAVA is safe mode of ventilation. The majority of studies have shown neither significant adverse events nor a difference in the rate of intraventricular hemorrhage, pneumothorax, or necrotizing enterocolitis when compared to conventional ventilation[26,28]. These were retrospective case series and randomized crossover studies with very small sample size. There are no up-to-date trials addressing

long-term outcomes.

Potential problems with NAVA

NAVA assumes that the respiratory center of preterm infants is mature enough to drive the ventilator at all times, with an appropriate rate, a sufficient magnitude and optimal IT and ET. This assumption may not hold true especially in extreme preterm infant, or during sepsis, intraventricular hemorrhage (IVH) or severe illnesses. Preterm infants demonstrate an immature response to hypercapnia[29,30], a paradoxical respiratory depression induced by hypoxemia[31] and a pronounced apneic response to laryngeal stimulation[32]. This immaturity of the respiratory drive in preterm infants very commonly results in apnea and periodic breathing in this group of neonates. These infants often produce a very small Edi signal that prevents backup ventilation with the PC mode but without producing a sufficient PIP to provide effective ventilation. This inability to generate a strong Edi may also prevent the termination of breath when the minimum Edi does not fall below 70% of the peak causing an extremely high inspiratory time. In invasive NAVA, a switch to PS takes place when the Edi signal is absent but the pneumatic trigger is still present. It occurs also when there is major discrepancy between the neural and pneumatic respiratory rates. As PS and PC are an integral part of NAVA, it is therefore incorrect to claim that NAVA is totally independent of air leak especially in VLBWI who switch frequently to PS and backup PC because of their immature respiratory drive.

A Cochrane meta-analysis demonstrated that the combination of volume targeted ventilation with PC or SIMV was associated with a statistically significant reduction of severe IVH, hypocarbia, pneumothorax, and the duration of ventilation[26]. VLBWI ventilated with NAVA keep moving back and forth between neutrally adjusted ventilation and PC backup. The non-availability of volume targeted ventilation and the fact that PIP during the backup periods is manually set by the clinician constitutes a major disadvantage of NAVA, considering the established benefits of volume targeted ventilation and the current lack of demonstrated long-term benefit of NAVA.

Capturing a strong and stable Edi signal is essential, but unfortunately, as Edi catheters for neonates are manufactured in three sizes only (6F/49 cm, 6F/50 cm and 8F/100 cm) while the length of newborns at birth is generally between 28 to 58 cm, NAVA effectiveness may not be as good as it should be. Capturing pneumatic triggers is also essential to the use of NAVA since pressure support and pressure control backup are required in case of apnea or discrepancy between pneumatic and neural triggers. Unfortunately, the flow sensor in Servo I and Servo U, which are the only ventilators providing NAVA, is located inside the machine and far from the ETT. As a result VLBWI are unable to constantly generate enough flow to compensate for the compliance of the tubing in order to trigger the ventilator. We have noticed that these patients frequently receive mandatory non-triggered breaths when they switch to the PC backup mode caused by either displacement of Edi catheter, or apnea or major discrepancy between pneumatic and neural triggers. Using a Y-peripheral flow sensor may provide a solution but must be purchased separately. The only two ventilators that offer the possibility of NAVA (servo I and Servo U) do not provide leak compensation or high frequency ventilation. This lack of versatility may become a limitation when volume targeted ventilation is required or when the clinical condition requires a change to high frequency oscillatory ventilation.

RECOMMENDATIONS

The place of NAVA in the management of respiratory distress in VLBWI is still not yet clear. We suggest that non-breathing infants should not be placed on NAVA because they do not produce a strong and consistent Edi signal to drive the ventilator. On the other hand, spontaneously breathing infants often do well on CPAP and do not require any type of ventilation. In our opinion, volume targeted ventilation in combination with PC should be the default mode of ventilation of VLBWI because it has been shown to reduce severe intraventricular hemorrhage, pneumothorax, hypocarbia and the duration of ventilation. As pressure control backup is very common in NAVA and since the long term benefits of NAVA have not yet been demonstrated in randomized trials, we recommend not to use NAVA in the first week of life in extreme low birth infants who have immature respiratory drive causing apnea and who already are at high risk of intraventricular hemorrhage and volume trauma. Larger and older infant can benefit from NAVA when they are able to generate a strong Edi activity. As NIV NAVA is presently the only ventilator mode that allows effective triggering despite the large air leak associated always present with nasal ventilation, we believe that it should be tried as a first option whenever possible. Patients with severe CLD and those who have received heavy sedation for long period may have a disuse atrophy of diaphragmatic muscle fibers and could benefit from progressive loading with NAVA to exercise their diaphragm and prepare them for possible extubation.

Future large size randomized controlled trials should be established to compare NAVA with volume targeted and pressure controlled ventilation in newborns with mature respiratory drive. Future studies should also compare NIV NAVA and biphasic CPAP or high flow nasal cannula to demonstrate if NIV-NAVA can prevent endotracheal intubation without causing abdominal distension or increasing the rate of necrotizing enterocolitis. As most previous studies and trials were not sufficiently large and did not include long-term patient oriented outcomes, multicenter, randomized, outcome trials are needed to determine whether NAVA is effective in avoiding intubation, facilitating extubation,

decreasing time of ventilation, reducing the incidence of CLD, decreasing length of stay, and improving long-term outcomes such as the duration of ventilation, length of hospital stay, rate of pneumothorax, CLD and other major complications of prematurity.

In order to prevent barotrauma, we recommend that the next generations of NAVA equipment for neonatal use should enable automatic setting of ventilatory parameters in the backup PC mode based on the values generated by NAVA. We believe that they should also include an upper limit to the IT as in conventional ventilation and that the manufacturers of Edi catheters should make smaller sizes available for extreme low birth weight infants. We also recommend that newly developed NAVA ventilators also include leak compensation, high frequency ventilation option as well as a peripheral flow sensor because it is essential for all modes of conventional ventilation as well as NAVA.

CONCLUSION

NAVA ventilation utilizes the patient's neural respiratory drive to synchronize ventilatory support on a breath-by-breath basis based on the infant's ongoing needs. It allows preterm neonates to use physiologic feedback to control ventilation and enhance comfort for each breath. The Edi signal provides the clinician with previously inaccessible information about central respiratory drive useful for both weaning and diagnostics, with infants informing the neonatologist of what support they need, directing both the timing and depth of their breathing pattern. NAVA appears to work well in neonates but if it makes a difference in outcomes in this population has not been established so far. The remaining challenge for neonatal health care providers is the steep and prolonged learning curve for the application of NAVA.

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Accurate diagnosis of myalgic encephalomyelitis and chronic fatigue syndrome based upon objective test methods for characteristic symptoms

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Abstract

Although myalgic encephalomyelitis (ME) and chronic fatigue syndrome (CFS) are considered to be synonymous, the definitional criteria for ME and CFS define two distinct, partially overlapping, clinical entities. ME, whether defined by the original criteria or by the recently proposed criteria, is not equivalent to CFS, let alone a severe variant of incapacitating chronic fatigue. Distinctive features of ME are: muscle weakness and easy muscle fatigability, cognitive impairment, circulatory deficits, a marked variability of the symptoms in presence and severity, but above all, post-exertional "malaise": a (delayed) prolonged aggravation of symptoms after a minor exertion. In contrast, CFS is primarily defined by (unexplained) chronic fatigue, which should be accompanied by four out of a list of 8 symptoms, *e.g.*, headaches. Due to the subjective nature of several symptoms of ME and CFS, researchers and clinicians have questioned the physiological origin of these symptoms and qualified ME and CFS as functional somatic syndromes. However, various characteristic symptoms, *e.g.*, post-exertional "malaise" and muscle weakness, can be assessed objectively using well-accepted methods, *e.g.*, cardiopulmonary exercise tests and cognitive tests. The objective measures acquired by these methods should be used to accurately diagnose patients, to evaluate the severity and impact of the illness objectively and to assess the positive and negative effects of proposed therapies impartially.

Key words: Myalgic encephalomyelitis; Chronic fatigue syndrome; Symptoms; Diagnosis; Disability; Impact

Core tip: The diagnostic criteria for myalgic encephalomyelitis (ME) and chronic fatigue syndrome (CFS) define two distinct clinical entities. Cognitive impairment and post-exertional "malaise" (a long-lasting aggravation of typical symptoms, *e.g.*, muscle weakness and cognitive "brain fog", after minor exertion) are obligatory for the diagnosis ME, while chronic fatigue is the only mandatory symptom for the diagnosis CFS. There is debate about the nature and severity of the symptoms in ME and CFS. For clinical and research purposes it is essential to accurately diagnose patients using objective tests for characteristic symptoms if possible. This article reviews accepted methods to assess various distinctive

symptoms of ME and CFS.

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INTRODUCTION

There is debate about various aspects of myalgic encephalomyelitis (ME) and chronic fatigue syndrome (CFS), including the nature of the symptoms, the etiology, the pathophysiology and presumed effective interventions, *e.g.,* cognitive behavioural therapy (CBT) and graded exercise therapies (GET)[1].

In light of the dispute about the origin of the symptoms, it is essential to assess the presence and severity of characteristic symptoms, and the impact and the disability in ME and CFS impartially as much as possible[2]. In the context of disability, it is important to establish physiological limitations in a specific patient objectively[3], independently of ones view on the etiology and the pathophysiology of ME and CFS.

To date diagnosis, symptom assessment and patient selection criteria of research studies of ME and CFS are often based upon self-report, questionnaires and subjective measures, *e.g.,* fatigue severity and impact. However, well-accepted methods can provide objective measures which can be used to diagnose patients more accurately. This article reviews relevant methods in this context.

ME/CFS is often initiated by an infection or another immunological insult[4]. Full recovery from ME/CFS seems rare (5%[5], 12%[6]). A long-term follow-up study[7] found that people who remitted from ME/CFS had non-significant differences in impairment on 17 out of 23 outcomes compared to those who maintained a CFS diagnosis. So, even patients who don't meet a CFS diagnosis anymore will not return to their premorbid level of functioning. ME/CFS has a greater negative impact on functional status and well-being than other chronic diseases, *e.g.,* cancer or lung diseases[8], and is associated with a drastic decrement in physical functioning[9]. In a comparison study[10] ME/CFS patients scored significantly lower than patients with hypertension, congestive heart failure, acute myocardial infarction, and multiple sclerosis (MS), on all of the eight Short Form Health Survey (SF-36)[11] subscales. As compared to patients with depression, ME/CFS patients scored significantly lower on all the scales, except for scales measuring mental health and role disability due to emotional problems, on which they scored significantly higher. Looking at several studies[12-16] the financial consequences of ME/CFS for the individual patient and the economic impact on society are often very profound.

This article aims to: (1) compares the diagnostic criteria for ME and CFS; and (2) reviews well-accepted methods to assess characteristic symptoms of ME and CFS objectively.

DIAGNOSIS

Although ME, CFS and post-viral fatigue syndrome are used interchangeably[17], the case criteria for ME[18] and CFS[19] define two distinctive clinical entities[1], delineating partially overlapping and partially disjoint patient populations (Figure 1).

ME, a neurological disease[20,21], has been described in the medical literature since 1934 under various names[22], *e.g.,* epidemic neuromyasthenia and atypical poliomyelitis, often on account of outbreaks[23-25]. Characteristic symptoms of ME, classified as a disease of the nervous system by the WHO since 1969[26], are: muscle weakness, neurological dysfunction, especially of cognitive, autonomic and neurosensory functions; variable involvement of the cardiac and other systems; a prolonged relapsing course; but above all general or local muscular fatigue after minimal exertion with prolonged recovery times (post-exertional "malaise")[20].

The clinical entity CFS was introduced in 1988[27] and redefined in 1994[19]. The diagnosis CFS is primarily based upon the ambiguous notion "chronic fatigue"[28,29]. According to commonly used criteria for CFS[19] "chronic fatigue" must be accompanied by at least 4 out of 8 symptoms, *e.g.,* tender lymph nodes and muscle and joint pain. However, 5 of the 8 "minor" symptoms, *i.e.*, headaches, lymph node pain, sore throat, joint pain, and muscle pain, do not differentiate people with melancholic depression group from healthy controls[30]. The CFS criteria[19] by definition select a heterogeneous population of people with "chronic fatigue"[31-34].

The diagnostic criteria for ME[18] and CFS[19] define distinctly nosological entities, since cognitive impairment and post-exertional "malaise", obligatory for the diagnosis ME, are not mandatory for the diagnosis CFS, and the diagnosis ME doesn't require "chronic fatigue". The distinction between ME and CFS is illustrated by a study[35] which found that 60% of the "less severe CFS" patents reported post-exertional "malaise" and 45% reported cognitive impairment. This implies that many "less severe" CFS patients don't fulfil the original[20] or new[18] criteria for ME. Looking at relevant studies[36-39] ± 30%-50% of subjects meeting the CFS criteria[19] seem to fulfil the more stringent International Consensus Criteria (ICC) for ME[18]. How many ME/ICC[18] patients don't meet the CFS[19] criteria is unknown, since almost all studies until now applied case definitions sequentially, *i.e.,* used other diagnostic criteria on a patient population preselected by chronic fatigue or CFS criteria[40]. In a recent study[41] ME/ICC[18] patients reported significantly more severe disability across all domains of the World Health Organisation Disability Adjustment Schedule 2.0[42] ($P < 0.05$), when compared to patients only fulfilling the criteria for

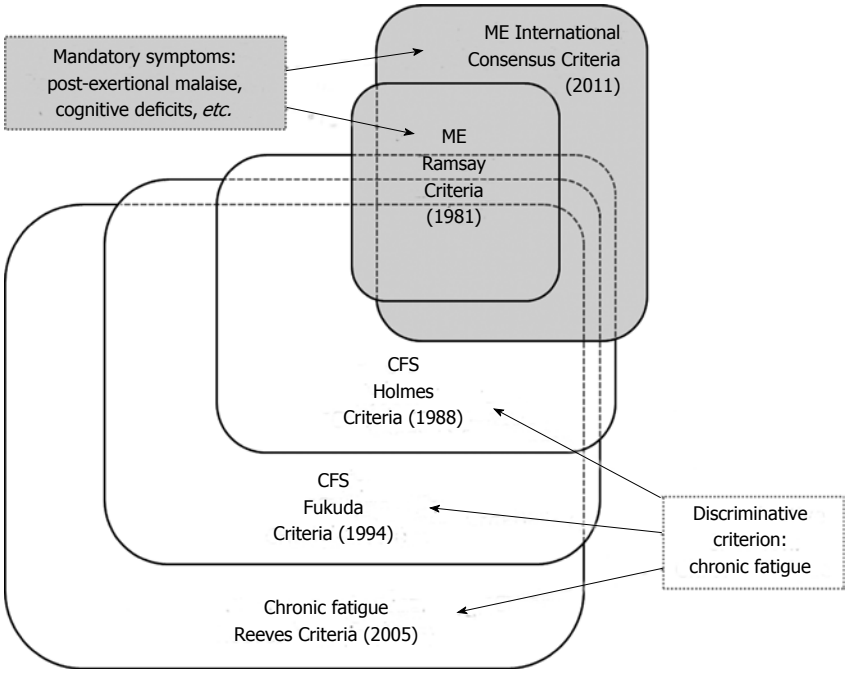

Figure 1 Myalgic encephalomyelitis *vs* chronic fatigue syndrome: Two distinct diagnostic entities. ME: Myalgic encephalomyelitis; CFS: Chronic fatigue syndrome.

Table 1 International Consensus Criteria for myalgic encephalomyelitis[18]

Post-exertional neuro-immune exhaustion:
A pathological inability to produce sufficient energy on demand with prominent symptoms primarily in the neuroimmune regions
Neurological impairments
At least one symptom from three of the following four symptom categories:
Cognitive impairments (information processing and short-term memory)
Pain (*e.g.*, headache, muscle, joint, abdominal and/or chest pain)
Sleep disturbance (disturbed sleep patterns and unrefreshing sleep)
Neurosensory, perceptual and motor disturbances
Immune, gastro-intestinal and genitourinary impairments
At least one symptom from three of the following five symptom categories:
Flu-like symptoms, *e.g.*, sore throat and tender lymph nodes
Susceptibility to viral infections with prolonged recovery periods
Gastro-intestinal tract complaints, *e.g.*, irritable bowel syndrome
Genitourinary complaints: *e.g.*, nocturia
Sensitivities to food, medications, odours or chemicals
Energy production and - transportation impairments
At least one of the following four symptoms:
Cardiovascular symptoms, *e.g.*, (delayed) orthostatic intolerance
Respiratory problems, *e.g.*, air hunger and fatigue of chest wall muscles
Loss of thermostatic stability, *e.g.*, sweating episodes or feverish feeling
Intolerance of extremes of temperature

CFS[19]. Another study[43] supports the notion that the ICC criteria for ME[18] identify patients with greater functional impairment and move severe physical, mental, and cognitive symptoms than patients who only meet the Fukuda criteria for CFS[19].

Diagnostic criteria applied (Figure 1) are crucial, not only because of the sensitivity and specificity[44-46] of the criteria[19,47], *e.g.*, resulting into the inclusion of people with psychiatric disorders[48], but also for a judgment about the effects of proposed effective interventions in specific patient populations[49-53].

Clinical assessment has shown to be essential for an accurate diagnosis and establishing prevalence rates. A recent study[54] for example observed that the pooled prevalence of CFS[19] was substantially higher for self-reporting assessment (3.28%, 95%CI: 2.24-4.33) than for clinical assessment (0.76%, 95%CI: 0.23-1.29).

In conclusion, ME[18] (Table 1) is not equivalent to CFS[19] (Table 2) or incapacitating chronic fatigue[55] (Table 3). While chronic fatigue is a common complaint, CFS[19] is a relatively rare condition (prevalence rate: 0.19%[38], 0.20%[58]). The prevalence of ME (CFS), as defined by more strict criteria[59], is even lower: 0.11%[38].

CHARACTERISTIC SYMPTOMS

ME/CFS patients often report a plethora of symptoms, which can vary in number and severity among individual patients and fluctuate within an individual over time, possibly as a result of daily activity[60]. Symptoms often reported by ME/CFS patients are post-exertional "malaise", cognitive deficits ("brain fog"), "fatigue" (lack of energy), muscle weakness, (muscle and/or joint) pain, impaired sleep, a new type of headaches, stress intolerance, orthostatic intolerance and visual symptoms[61].

Objective assessment of characteristic symptoms

Various typical symptoms, *e.g.,* post-exertional "malaise" and muscle weakness, can be quantified objectively using accepted, reproducible methods[1] (Table 4), while others symptoms, *e.g.,* fatigue, cannot be evaluated objectively due to their nature.

Due to the multi-systemic nature of ME/CFS, objective assessment of symptoms and disability in ME/CFS involves various medical specialists, *e.g.,* cardiologists, neuropsychologists, exercise physiologists,

Table 2 Fukuda *et al*[19] Diagnostic Criteria for chronic fatigue syndrome

Primary symptom:
Clinically evaluated, unexplained, persistent or relapsing chronic fatigue
That is of new or definite onset; is not the result of ongoing exertion
That is not substantially alleviated by rest; and
That results in substantial reduction in previous levels of occupational, educational, social, or personal activities
Secondary symptoms:
The concurrent occurrence of four or more of the following symptoms, all of which must have persisted or recurred during 6 or more consecutive months of illness and must not have predated the fatigue:
Self-reported impairment in short-term memory or concentration severe enough to cause substantial reduction in previous levels of occupational, educational, social, or personal activities
Sore throat
Tender cervical or axillary lymph nodes
Muscle pain
Multi-joint pain without joint swelling or redness
Headaches of a new type, pattern, or severity
Unrefreshing sleep
Post-exertional malaise lasting more than 24 h

Table 3 Empirical case definition for chronic fatigue (syndrome)[47]

Fatigue:
A score
⩾ 13 (out of 20) on the general fatigue or
⩾ 10 (out of 20) on the reduced activity
subscales of the multidimensional fatigue inventory[56]
Functional impairment:
A score
⩽ 70 (out of 100) on the physical function, or
⩽ 50 (out of 100) on role physical, or
⩽ 75 (out of 100) on the social function, or
⩽ 66.7 (out of 100) on the role emotional
subscales of the medical outcomes survey short form-36 (SF-36)[11]
Secondary symptoms:
⩾ 4 of the following 8 symptoms:
Impaired memory or concentration
Unrefreshing sleep
Headaches
Muscle pain
Joint pain
Sore throat
Tender cervical nodes and
Unusual post exertional fatigue
And
A score of ⩾ 25 (out of 128)
On the Symptom Inventory Case Definition subscale[57]

and endocrinologists. The exclusion of psychiatric diseases[18,19] and assessment of comorbid psychological disorders requires the input of psychologists/psychiatrists.

Table 4 denominates tests that have demonstrated to yield aberrant results in ME/CFS. However, Table 4 should not consider to be exhaustive. Due to the heterogeneity of the ME/CFS patients population[1,34,88], not all patients will experience all symptoms, *i.e.*, not all tests will show deviant results in all ME/CFS patients. Nevertheless, to assess the clinical status, the severity of the illness, and the disability of an individual patient impartially, patients should be subjected to the abovementioned and other objective tests as much is feasible.

Lack of energy: physical weakness and "fatigue"

ME/CFS is often incorrectly considered to be equivalent to chronic fatigue. "Fatigue" in ME/CFS is a multi-dimensional entity that is distinct from the generalized form of fatigue experienced by the general population[28]. Fatigue in ME/CFS encompasses at least five dimensions: a lack of energy resources needed for basic daily functioning, "brain fog", post-exertional "malaise", a "wired feeling" when very tired, and a flu-like feeling[28]. While these latter two aspects of "fatigue" are subjective due to their nature, the first three dimensions can be assessed more objectively. This paragraph focuses on "lack of energy", while "brain fog" (neurocognitive deficits) and post-exertional "malaise" will be discussed in the next paragraphs.

Cardiopulmonary exercise testing (CPET) is regarded to be an accurate method for assessing functional capacity[62,89]. The (maximum) oxygen uptake (O2) measured at a CPET is associated with the concept of Metabolic Equivalent of Task (MET): the energy cost of a physical activity compared to the energy produced by an average person seated at rest. By definition, 1 MET is equivalent to an oxygen utilization of 3.5 mL O_2/kg per minute. The functional capacity established by a CPET can be set against the metabolic requirements[90] of self-care tasks essential for fundamental functioning (Basic Activities of Daily Living, ADLs) and activities crucial to live independently from others (Instrumental Activities of Daily Living, IADLs)[91].

Although contradicted by some studies, *e.g.*,[92,93], various studies, *e.g.*,[94-98], implicate that the "lack of energy" experienced by ME/CFS patients is reflected in the performance levels at a CPET: a low maximum workload (Wmax) and oxygen uptake (VO2max) and a reduced anaerobic threshold (W AT) and corresponding oxygen uptake (VO2 AT), when compared to sedentary controls. When looking at the "high" mean performance levels of patients in some studies, *e.g.*,[92,99], contradictory findings are likely due to heterogeneity of the patient samples, *e.g.*, participation rates of "severe" *vs* "less severe cases".

Whether or not the exercise capacity is decreased or not in an individual patient should be assessed impartially by a CPET. Objective measures should be employed to establish the degree of effort during exercise, *e.g.*, the respiratory exchange ratio at peak exertion (RERmax). According to well-accepted criteria[62,63] a RERmax > 1.10 indicates excellent effort, while a RERmax < 1.0 reflects submaximal effort. Although (some) patients seem to be able to perform at a level comparable to that of sedentary controls once-off, a CPET often has profound negative effects on the

Table 4 Symptoms and tests to assess the disability in myalgic encephalomyelitis/chronic fatigue syndrome objectively

Symptoms	Tests	Ref.
Lack of energy: physical weakness and "fatigue"	CPET 1: workload and oxygen uptake at exhaustion and at the anaerobic threshold	[62,63]
Cognitive impairment	Specific neuropsychological tests	[64-67]
Post-exertional "malaise"		
Physical effects	Repeated CPETs 1, 24 h apart	
Cognitive effects	Specific neuropsychological tests before and after a CPET or before and during a tilt table test Repeated neuropsychological tests	
Muscle weakness	Examination of the muscles (power, endurance, recovery)	[68-71]
Orthostatic intolerance	Tilt-table test	[72-74]
Defective stress response	Hormonal investigation (HPA axis, thyroid) in rest, at specific moments, *e.g.*, at wakening, and during the day, after provocation, *e.g.*, by adrenocorticotropic hormone and insulin, and in response to an exercise test or psychological stress test	[75-78]
Sleep impairment	Polysomnographic investigation (EEG)	[79-81]
	Maintenance of wakefulness test	[79,82,83]
	Multiple sleep latency test	[79,82,83]
Visual symptoms	Useful field of view tests	[84,85]
	Eye movement tests	[86,87]

CPET: Cardiopulmonary exercise test.

exercise capacity 24 h later at a second CPET (see Post-exertional "malaise").

A "lack of energy" seems to be accompanied by hypovolemia (low blood volume)[98,100], low cardiac mass[101-103] and reduced cardiac function[100,104]. Some studies implicate interrelations between hypovolemia and low cardiac output[100] and between hypovolemia and (maximum) oxygen uptake[105]. A reduction of the exercise capacity seems to be associated with typical immunological abnormalities in ME/CFS, including immune activation and immune dysfunction[106-108].

In addition to a reduced exercise capacity, the "lack of energy" of ME/CFS patients seems to manifest itself in post-exertional malaise[109], muscle weakness[110] and orthostatic intolerance[111], which will be discussed in separate paragraphs.

Cognitive impairment

A second characteristic symptom of ME/CFS is cognitive impairment ("brain fog")[39]. Several studies, *e.g.*,[112-118], have established a wide range of neurocognitive deficits in ME/CFS. In addition, various studies have observed neurological aberrations[119-121], *e.g.*, reduced white[122-124] and grey[123,125,126] matter volume, electroencephalography (EEG) abnormalities[127], hypoperfusion of the brain[128-130], hypometabolism[131,132], neuro-inflammation of widespread brain regions[133], increased fractional anisotropy in the right arcuate fasciculus and, in right-handed patients, of the right inferior longitudinal fasciculus[124], and spinal fluid abnormalities[134,135]. A relationship between neurological anomalies and cognitive symptoms has also been observed[136-138]. Some findings indicate that the neurocognitive problems are induced or intensified by exercise[97,139] and an upright (orthostatic) position[140]. Cognitive impairment seems to be more severe in sudden onset-ME/CFS[141,142].

ME/CFS patients can present with moderate to large deficits in simple and complex information processing speed (attention, memory and reaction time)[143], in tasks which require working memory over a sustained period of time[143,144], in tasks which necessitate (simultaneous) processing of complex information[116,117] and in conflict-monitoring tasks (interference control)[145]. Specific cognitive deficits, reduced exercise capacity, decreased muscle power (strength and endurance) and immunological aberrations, *e.g.*, inflammation, seem to be interrelated[146,147].

Cognitive impairments can be identified, but only if the appropriate measures are used[114]. This important observation is confirmed by a meta review of 50 studies and 79 tests[143]. All tests for assessing attention, including attention span and working memory, showed significant deficits in ME/CFS. The effect sizes for most word list learning and recall tests were significant, but some tests seem more sensitive to memory deficits in ME/CFS than others. Reaction time is substantially impaired for responses to both simple and complex (choice) stimuli. Only two of the five tests used to assess movement times revealed significant group differences. Most tests for visuospatial ability, verbal abilities and language, cognitive reasoning and flexibility, and global functioning didn't yield significant group differences. In order to determine cognitive impairment objectively, ME/CFS patients should be subjected to neuropsychological tests[64-67] aimed at the abnormalities found in ME/CFS patients, *e.g.*, attention and memory[112,116,143].

Cognitive deficits don't seem to be related to "fatigue" or comorbid depression[148,149]. Goedendorp *et al*[150] have suggested that low cognitive test scores are due to underperformance, but this view is based upon the subjective premise that ME/CFS has not proven to be a cognitive disorder[151]. Objective measures indicate high levels of effort and an intention to do well during neurocognitive testing[152].

Post-exertional "malaise": physical and mental

Post-exertional "malaise" has been defined as "a pathological inability to produce sufficient energy on demand"[18], resulting into a (delayed) increase of typical symptoms, *e.g.*, weakness, muscular and/or joint pain, cognitive deficits, after a minor physical or mental exertion, with prolonged "recovery" times[109,153].

Looking at the research, post-exertional malaise in ME/CFS can present itself in several forms, including a decline in physiological exercise capacity at a second exercise test 24 h later[94,154], cognitive impairment

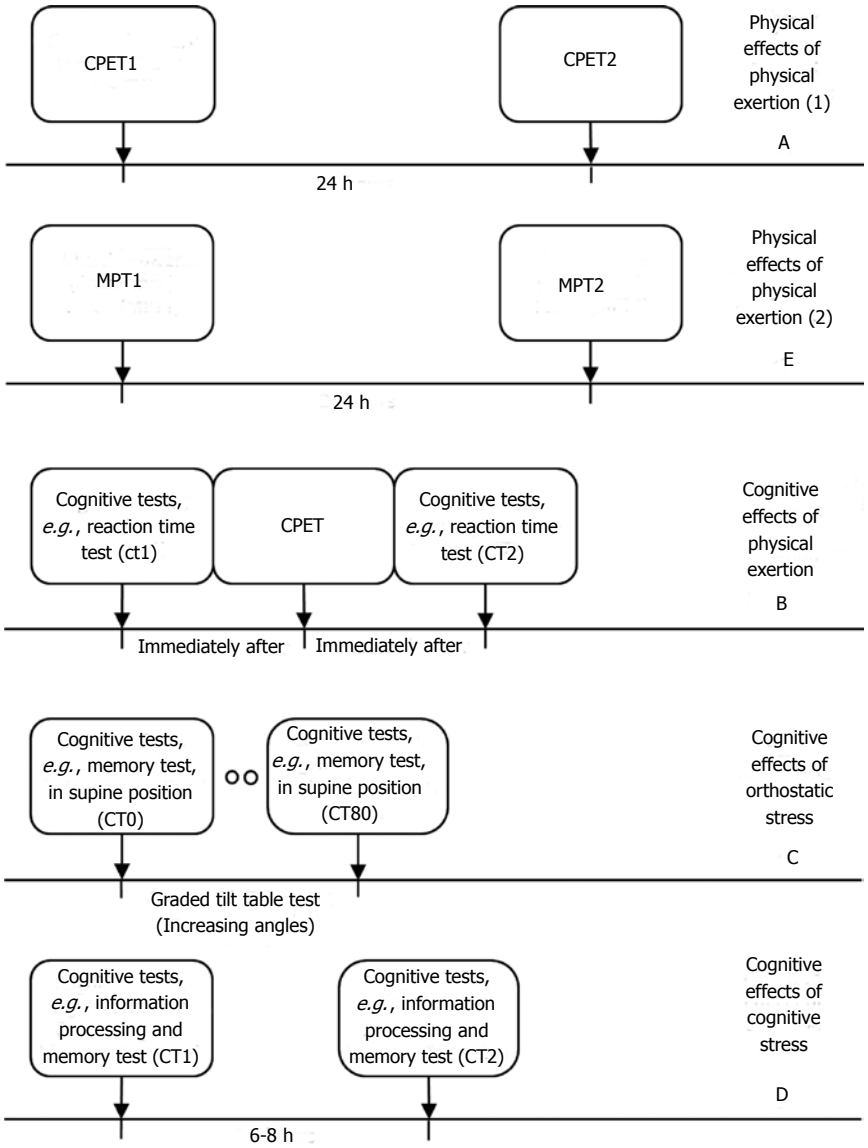

Figure 2 Objective tests for post-exertional malaise. CPET: Cardiopulmonary exercise test; MPT: Muscle power test.

induced or intensified by exercise[97,139] and orthostatic stress[140], and cognitive deficits due to mental exertion, *e.g.,* cognitive testing[155]. These various dimensions of post-exertional "malaise" can be assessed by combining CPETs, tilt-table and specific cognitive tests (Figure 2). The (long-lasting) physical effects of physical exertion (Figure 2A) can be evaluated objectively by subjecting a patient to two CPETs until exhaustion separated by 24 h[156]. The cognitive effects of physical exertion (Figure 2B) can be assessed impartially by comparing the cognitive performance, *e.g.,* simple and choice reaction times, before and immediately after a CPET, the cognitive effects of orthostatic stress (Figure 2C) can be established by subjecting patients to cognitive tests at various stages of a graded tilt table test, while the mental effects of cognitive exertion (Figure 2D) can be assessed objectively by exposing a patient twice to the same cognitive tests with several hours rest in-between. The prolonged negative effect of muscle contractions on muscular strength (Figure 2E), another aspect of the physical dimension of post-exertional "malaise", will be discussed in the paragraph Muscle weakness.

Physical effects of physical exertion: A profound decrease in the exercise capacity at a second CPET 24 h after the first CPET seems typical for ME/CFS and is neither observed in sedentary healthy controls nor in patients with other diseases[154,157]. The "exercise intolerance" in ME/CFS can be reflected in significantly lower oxygen uptake and performance levels at exhaustion (VO2max and Wmax) or at the anaerobic threshold (VO2 AT and W AT) at the second CPET[154]. In contrast, the first CPET appears to have a positive effect on the anaerobic threshold in sedentary controls at the second CPET[94]. Due to the first CPET the anaerobic threshold can decrease to a level below 5 METS; a level at or below that which is required by many job-related activities and IADLs[158]. Since many daily activities fall into the 3-5 MET energy range, persons with ME/CFS will exacerbate symptoms simply by completing normal daily activities[158]. A recent study[157] observed that VO2max at the first exercise test was reduced in

Table 5 Adverse effects of a CPET (CPET1) on the performance levels at a second CPET (CPET2) 24 h later: An example

	CPET Day 1	CPET Day 2
Rest		
Heart rate	88	80
Oxygen uptake (VO2min)	6	6
Anaerobic threshold		
Heart rate (HR AT)	105	89
Oxygen uptake (VO2 AT)	11	9
Workload (W AT)	54	35
Exhaustion		
Heart rate (HRmax)	151	131
Oxygen uptake (VO2max)	23	22
Workload (Wmax)	159	133

CPET: Cardiopulmonary exercise testing.

ME/CFS (mean ± SD: 21.9 ± 4.75 mL/k per min), that all patients showed clinically significant decreases in either VO2max and/or oxygen uptake at the ventilatory threshold (VO2 VT) at the second CPET, and that a classification of impairment[159] based on the VO2max or VO2 VT of the first CPET would result in overestimation of functional ability for 50% of the patients.

A real-life example of the effect of a CPET on the performance levels at a second CPET 24 h is summarized in Table 5 (male patient, 45 years, 65 kg).

The VO2max at day 1 was 23 mL/min per kilogram, while the anaerobic threshold was reached at a workload level of 54 Watt (W). The corresponding oxygen uptake (VO2 AT) was 11 mL/min per kilogram. Washing the floor requires 58 W and walking at a speed of 5 km/h 56 W[160], which implies that the anaerobic threshold (AT) is reached just by doing light household activities or low-speed walking. On the second CPET the AT declined to a workload of 35 W, which is equivalent to the energy cost of ironing (35 W) and walking at 3 km/h (32 W)[160]. The oxygen uptake at the anaerobic threshold (VO2 AT) has decreased to 9 mL/min per kilogram on day 2. The difference between the heart rate at the anaerobic threshold and the heart rate at rest is only 9 bpm at day 2. This example perfectly illustrates the (prolonged) negative effects of exertion in ME/CFS.

Mental (cognitive) effects of physical exertion: Although research studies into the effects of physical exercise on cognitive performance are scarce, there are several indications that exercise has a (durable) negative effects on cognitive functioning, *e.g.,* focused and sustained attention[161], simple reaction time and choice reaction times[97] and accuracy at the Symbol Digit Modalities Test, Stroop Word Test and Stroop Color Test[139]. This negative effect seems to be the opposite of the effect of exercise on cognitive performance in sedentary controls[139]. The negative impact of physical exertion on cognitive functioning could be mediated by reduced prefrontal cortex oxygenation during and after exercise[98] and/or diminished cardiovascular response to cognitive stress[162]. The effects of physical exertion on cognitive impairment can be assessed by subjecting a patient to specific cognitive tests[114,143] 0-24 h after a CPET and comparing the results on these tests with the pre-exercise test scores.

Mental (cognitive) effects of orthostatic stress: A subgroup of patients present with (delayed) orthostatic intolerance[163], as implicated by a substantially increased heart rate and/or reduced blood pressure in an upright position (POTS: postural tachycardia syndrome, respectively postural hypotension)[74]. Orthostatic symptoms are independently associated with functional impairment[164]. Orthostatic stress seems to impair working memory and information processing, as indicated by a deterioration of scores and reaction times on the N-back test as orthostasis progresses[140,165]. This phenomenon could be related to reduced neuronal activated cerebral blood flow velocity during orthostatic stress[165]. To assess the effects of orthostatic stress objectively, the patient should be subjected to cognitive tests at various angles during tilt-table testing. The N-back test could be a suitable cognitive test, since studies[140,165] have found that while N-back outcome in controls decreased with the value of N, the score was independent of tilt angle, while N-back outcome in ME/CFS patients also decreased with the value of N, but deteriorates as the tilt angle increases. A recent study[166] found that upright tilting caused a significant increase in the N-back normalized response time and a profound drop in cerebral blood flow velocity.

Mental (cognitive) effects of cognitive stress: Although scarcely investigated, there are indications that cognitive stress induces long-lasting mental/cognitive effect in ME/CFS. As mentioned in the paragraph Cognitive Impairment, "recovery" from a 3-h lasting cognitive test to pre-test levels of mental energy seems to take much longer in ME/CFS patients[155]. This phenomenon could be associated to a greater mental and neurological effort to process information as effectively as healthy controls[136-138,167]. A recent study[168] observed significant differences between self-reported levels of general, mental, and physical fatigue[58] and depression[169] before and two days after a cognitively fatiguing task[67]. Whether the effects of cognitive stress encompasses a mental dimension in a specific patient, could be assessed objectively by subjecting a ME/CFS patient to cognitive tasks 6-8 h after a fatiguing cognitive test battery and comparing the first and second scores on specific cognitive tests[114,143].

Muscle weakness: Many patients report muscle weakness[30,35,44]. According to a recent study[170] muscle recovery is closely related to cognitive deficits (see cognitive impairment). Several studies indicate reduced muscle strength and endurance and prolonged recovery from muscle contractions in ME/CFS. One study[171] for example found that the hand grip strength of patients

was significantly (26%) less than sedentary controls and that the maximum voluntary contraction (MVC) force in the patient group significantly reduced to 83% of the low baseline strength after 50 contractions with 10 s and 50 contractions with 5 s rest between trials. This study also observed deviant EEG-recorded brain signals in controlling voluntary muscle activities, especially when the activities induce fatigue. Another study[172] observed that ME/CFS patients were able to sustain a 10 kg handgrip contraction for less time than healthy controls and that the mean of handgrip contraction strength was substantially lower in ME/CFS patients when compared to healthy controls. A third study[110] found that the maximum twitch interpolated voluntary isometric contraction force of the quadriceps muscle of the dominant leg was significantly lower in ME/CFS patients (interquartile range, IQR: 234N-386N) than in sedentary controls (IQR: 364N-518N). In addition to reduced muscle strength and endurance, recovery of voluntary muscle contractions seem to be prolonged. This is illustrated by a study[173] in which patients and sedentary controls were subjected to an experiment involving 18 MVCs with a 50% duty cycle (10 s contraction, 10 s rest), followed by a recovery phase, lasting 200 min, in which the strength of the quadriceps muscle group was assessed, and a follow-up session 24 h post-exercise, involving three 10 s MVCs. The MVC forces in the control group were significantly higher than those of the ME/CFS patients, with a decline in force over the 18 contractions in both groups. Recovery was prolonged in the patient group, with a significant decline in the MVCs during the recovery phase and also at 24 h-post exercise (73% ± 9% of the initial force levels in patients *vs* 91% ± 7% in controls). In a recent study[174] patients exhibited lower isometric MVC levels for handgrip strength and slower and incomplete recovery in the 45 min after a fatiguing exercise (18 maximum contractions using a 50% duty cycle, 5 s contraction, 5 s rest), compared to both MS patients and sedentary healthy controls.

All in all, muscle weakness in ME/CFS seems to manifest itself into reduced muscle power, declining dynamic muscular endurance and the long-lasting recovery from repeated voluntary muscle contractions (Figure 2E). In order to assess muscle weakness and recovery from muscle exercise objectively and reliably the use of isokinetic and isometric dynamometers is essential[68-71,175-178].

Various studies have observed impaired skeletal muscle metabolism in ME/CFS, *e.g.,* decreased basal values of PCr/(PCr + Pi), increased pH levels during exercise, and low intracellular concentrations of ATP at exhaustion of a graded exercise of the right gastrocnemius muscle[179,180]. In addition to a significant prolongation (almost 4-fold) of the time taken by pH to recover to baseline after exercise, Jones *et al*[181] revealed the existence of two CFS[19] subgroups: patients with normal PCr depletion in response to a low-level voluntary contraction exercise, but with substantially increased intramuscular acidosis, and patients with low PCr depletion during exertion, generating abnormally low muscle power. An impaired cardiovascular response to standing, orthostatic intolerance, cardiac bioenergetic abnormalities, as implicated by low PCr/ATP values, and reduced muscle metabolism (longer PCr and ADP recovery times) seem to be interrelated[101].

Orthostatic intolerance: Orthostatic intolerance is accompanied by symptoms that arise or aggravate while standing, *e.g.,* light-headedness, blurred vision, fainting and syncope. Several studies[182-185] indicate orthostatic intolerance in ME/CFS patients or subgroups. Altered cardiovascular autonomic control and responses to orthostatic stress are associated with other typical symptoms, *e.g.,* cognitive deficits, and disability[186]. Since orthostatic intolerance is already present in the early stages of the disease[182,187] and CFS patients with POTS were have found to be significantly younger and to have a shorter length of illness than CFS patients without POTS[188], it seems unlikely that prolonged inactivity accounts for the orthostatic symptoms.

Orthostatic intolerance (in ME/CFS) seems associated with specific cardiovascular abnormalities in an upright position[111,189-191], *e.g.,* POTS and neurally mediated hypotension (NMH). These cardiovascular aberrations can be assessed using a tilt table test. The head-up tilt testing is considered a clinically useful diagnostic tool to assess susceptibility to orthostatic intolerance in patients with syncope, allowing reproduction of the patient's symptoms in a safe environment, under medical control[72,192]. With regard to deviant cardiovascular responses to orthostatic stress five types of abnormalities[74] can be distinguished (Table 6).

In a tilt table test a patient has to lie on a special table/bed, which gradually moves in posture from lying to an "upright position", *e.g.,* 70 degrees. Heart rate and blood pressures are monitored at various angles in order to establish hemodynamic abnormalities in a specific subject. It is relevant to note that the abovementioned aberrations seem to occur delayed and suddenly in ME/CFS[163,195]. So, in order to assess potential orthostatic abnormalities in a patient, the patient should remain in an "upright" position as long as possible, preferably longer than 15 min.

Defective stress response: Not only physical but psychological stress as well seems to intensify the symptoms in ME/CFS[196]. This phenomenon seems to be associated with hypothalamic-pituitary-adrenal (HPA) axis dysfunction[197,198], including hypocortisolism and deviant physiological responses to stress. HPA dysfunction in ME/CFS can manifest itself in reduced levels of stress hormones, *e.g.,* cortisol, at specific moments of the day[199,200] and aberrant diurnal production of specific hormones, *e.g.,* cortisol, cortisone and adrenocorticotropic hormone (ACTH)[201,202]; a blunted response to provocation, *e.g.,* by insulin[203], ACTH[204] or CRH[78]; a (long-lasting) deviant response

Table 6 Manifestations of orthostatic cardiovascular abnormalities

Abnormality	Definition
Orthostatic systolic hypotension	A fall in the systolic blood pressure of 20 mmHg or more[74,193]
Orthostatic diastolic hypotension	A fall in the diastolic blood pressure of 10 mmHg or more[74,193]
Orthostatic diastolic hypertension	A rise in dBP to 98 mmHg or more[74]
Orthostatic postural tachycardia	An increase in heart rate of 28[74]/30[194] beats per minute (bpm) or a pulse of more than 110[74]/120[194] bpm
Orthostatic narrowing of pulse pressure	A fall in the pulse pressure to 18 mmHg or less[74]

to psychological[205] or physical stress[205,206], and an enhanced sensitivity of the cellular immune system to glucocorticoids[207,208] and increased negative feedback of glucocorticoids to the HPA axis[209,210]. HPA axis dysfunction is not likely to be the primary cause of the illness, since HPA axis hypofunction, *e.g.,* hypocortisolism, is only present in a subgroup of patients[200,211], HPA axis abnormalities manifest themselves at a later stage of the illness[212-214] and hydrocortisone/fludrocortisone seem to have limited[215] or adverse[216] effects.

HPA axis dysfunction in ME/CFS can result into (1) low basal levels of ACTH; (free and total) cortisol (according to gas chromatography-mass spectrometry and high-performance liquid chromatography are considered to be the golden standard for assessing cortisol levels[78]), DHEA/DHEAS and noradrenalin at specific moments of the day, *e.g.,* at awakening; (2) reduced synthesis of ACTH and cortisol during the day; and (3) blunted HPA axis responses to "provocation", exercise or psychological stress. Tests[75,217] to assess HPA axis dysfunction objectively should be aimed at these aberrations.

ME/CFS has also been associated with thyroid dysfunction[218]. This finding is in line with inflammation-mediated loss of thyroid function[219-221]. Thyroid dysfunction can present itself in (1) low (free) thyroxine (T4) levels[222], due to decreased levels of thyroid stimulating hormone (TSH) secreted by the pituitary or a blunted response of the pituitary to TSH; (2) by reduced uptake of triiodothyronine (T3) and T4 by the cell[223,224]; (3) by diminished T4-T3-conversion, resulting into increased levels of reverse triiodothyronine (rT3)[225,226]; (4) by a diminished production of TSH and free T3 and T4 after administration of thyrotropin-releasing hormone (TRH)[227]; and/or (5) antithyroid microsomal antibodies[228]. Thyroid tests[229] could reveal if these aberrations are present in a particular patient.

Sleep impairment: Many patients report sleep disturbances[30,35,230,231], *e.g.,* insomnia, frequent awakenings, vivid dreams/nightmares and day/night reversal. Non-restorative sleep is the most specific and sensitive "minor" symptom[232] of CFS[19]. Sleep seems to be disturbed differently patterns in ME/CFS patients with and without comorbid fibromyalgia[233]. Abnormalities have been observed in reduced theta, sigma, and beta spectral power during the various sleep stages and shorter duration and higher frequency of transitions between the sleep stages[234-239].

Some methods to establish sleep dysfunction in ME/CFS objectively are: polysomnographic sleep investigation (EEG), aimed at the frequency of transitions between and the duration of sleep phases[233,237] and spectral power analysis[235,239], the maintenance of wakefulness and the multiple sleep latency test, although the latter two could be considered subjective, and not objective tests.

Visual symptoms: Patients often report visual symptoms, *e.g.,* problems with focusing, blurred vision and light insensitivity[240-242]. While various visual symptoms can be qualified as subjective, some aspects of the visual function can be assessed objectively. Abnormal visual attention, *e.g.,* conjunctive search (with divided and selective attention) and spatial cueing (selective attention with distraction)[243], can be assessed with the Useful Field of View test[84,85]. According to a recent study[244] dysfunctional eye movements in ME/CFS can present itself in reduced antisaccade focus accuracy and less precision and speed at smooth pursuing a target.

Subjective assessment of characteristic symptoms

While several symptoms can be assessed objectively, other characteristic symptoms[18,30] can't be quantified easily due to their nature. These symptoms include pain (muscle and joint pain, headaches, *etc.*), abdominal pain and other gastro-intestinal symptoms, "sickness behavior" (flu-like feeling, depression, *etc.*), intolerance of light (photophobia), sound (phonophobia) and odors (osmophobia), food and chemicals, and disturbed thermoregulation[18,30]. However, several of these symptoms could logically be explained by aberrations observed in ME/CFS patients or subgroups. Pain *e.g.,* could be the result of (1) inflammation[245,246]; (2) reduced oxidative metabolism[180,247], mitochondrial dysfunction[248,249] or damage[250,251]; (3) low cardiac output[101,185] and reduced blood and oxygen supply to the brain[98,129] and muscles[252,253], possibly leading to acidosis[181,254], accelerated glycolysis[180] and elevated lactate levels[132,255]; (4) central sensitisation[256], as a potential sequel of inflammation[257,258] and oxidative and nitrosative stress[259,260]; and (5) elevated pain receptors[261,262].

When assessing patients it is crucial to keep in mind that while various symptoms are obligatory for the diagnosis ME[18], they are not obligatory for to fulfil the CFS[19] diagnostic criteria. As argued, objective tests are to be preferred when possible. Nevertheless, when questionnaires and subjective measures are used to assess the clinical status of a patient, applying

minimum thresholds for the frequency and the severity of symptoms (*e.g.,* moderate severe about half of the time) can reduce the likelihood of possible misclassification of healthy persons and ME/CFS patients[61].

IMPACT

ME/CFS has a profound effect on the functional status[10,263] and life[264,265] of patients. As argued in this chapter, an objective assessment of the clinical status can quantify the severity of characteristic symptoms, *e.g.,* cognitive impairment, low physiological exercised capacity and the detrimental effects of exertion. However, next to the illness burden, patients experience serious medical, financial, social and psychological consequences of their illness, which can have a profound impact.

An objective assessment of symptoms could help to resolve the controversy around the nature and impact of ME/CFS within the medical profession. Patients for example often report negative experiences with health care workers[266]. Some medical professionals don't consider ME/CFS to be a legitimate illness[267]. Despite the neurological classification of ME/CFS[17,26] and various neuro-immunological abnormalities in ME/CFS observed repetitively[18,268], 84% of respondents in a survey of members of the Association of British Neurologists answered they did not consider ME/CFS to be a neurological condition[269]. According to a study[270] only half of the general practitioners (GPs) believed that ME/CFS actually exists. In a survey[271] patients reported that they felt that the doctors psychologized too much or trivialized the symptoms. Increasing physical activity had been recommend by doctors, but most of the respondents reported that this made them worse[271].

ME/CFS often has a huge impact on the occupational status[272] and income of patients[16], school attendance[273] and performance[265] of young patients, and the income of parents of children with ME/CFS[14]. A substantial proportion of ME/CFS patients, 50.1% according to[16], has to discontinue their employment due to their illness. Looking at the data of various studies[12,13,14] the average annual direct cost (medical costs, *etc.*) vary from $ 2342 to $ 8675, and the indirect cost (work productivity losses, disability reimbursements, services provided by family members, friends and others, *etc.*) vary from $ 8554 to $ 20000, for men: $ 23124[12,15,16]. Based upon the prevalence rates of[38] the direct and indirect cost of ME/CFS to the US society could be estimated at $ 8.5-$11 billion annually.

The prevalence of ME/CFS in children based upon a cross-sectional national sample among GPs was 0.111%[274], which is comparable with other prevalence figures[38]. The impact of ME/CFS on school attendance of children and adolescent is profound. On study for example found that 62% of children and young people aged under 18 years with ME/CFS, attended 40% of school or less[275]. Another study[274] observed that 45% of young patients with ME/CFS (aged 10 to 18 years) reported > 50% school absence during the previous 6 mo. A substantial subgroup of young patients, 29% of patients aged 12-18 years according to Bould *et al*[276], reports symptoms of comorbid depression, which seems to be associated with the degree of disability. The prognosis of children with CFS seems better in adolescents, *e.g.,* in CFS induced by infectious mononucleosis[277,278], but in both adults and adolescents the severity of the acute phase seems to be the sole predictor of the outcome[187,278].

ME/CFS also can have serious social and emotional consequences, *e.g.,* marginalisation, social isolation, stigmatisation and transformation of identity. Many patients with ME/CFS feel that their illness is not acknowledged as a legitimate illness within the social and medical context[271], and patients often report marginalization from family, friends, and medical professionals[279]. Not being able to be with friends or to attend school, makes adolescents with ME/CFS feel isolated, different and forgotten[280]. ME/CFS can also result into a transformation in identity[272,279] and values, expectations and life priorities[281]. In addition to destroying relationships and careers[272], ME/CFS also can disrupt self-perceptions[282]. Much of the stigma experienced by ME/CFS patients seems to originate from the associations with the name CFS[283], the lack of diagnostic biomarkers[284] and the absence of clear-cut etiologic models for ME and CFS[284]. Questioning the veracity of ME/CFS might represent a potent stressor in ME/CFS, and even coping methods thought to be useful in other conditions, are not associated with a reduction of distress among those with ME/CFS[284]. Doctors' beliefs can result into negative stereotyping of ME/CFS patients[285]. However, there don't seem to be major differences between the personalities of ME/CFS patients and patients with rheumatoid arthritis and the stereotype of ME/CFS patients as being "perfectionists with negative attitudes toward psychiatry" doesn't seem to be applicable[286].

DISCUSSION

ME/CFS is a serious disorder, which can have profound consequences on a patients' life and health status. In addition to the impact of the symptoms on everyday life, patients often disbelief, *e.g.,* when claiming disability related benefits[156]. Due to the fact that "chronic fatigue" is an ambiguous and subjective notion[28], that patients often report a plethora of symptoms which can fluctuate over time very rapidly, and that there are (yet) no clear-cut etiological models for ME and CFS, patients frequently encounter difficulties in proving their level of disability, which can have substantial financial consequences.

An objective assessment of core symptoms could not only impartially confirm the patients' self-reported disability[287], but could also contribute to reversal of other problems experienced by patients, *e.g.,* stigmatization and the attitude of medical professionals

towards the illness(es) and patients. In this context and in light of the controversy surrounding ME and CFS, it is essential to establish the functional (dis)abilities of a patient (output or functional consequences) objectively without an a priori judgment about the causes (the "black box": etiology and pathophysiology). However, establishing the functional impact objectively using well-accepted tests, *e.g.,* (repeated) exercise tests (CPETs), neurocognitive tests and tilt table tests, also could point towards the physiological origin of various symptoms, *e.g.,* post-exertional "malaise".

One very relevant limitation with regard to the objective assessment of symptoms relates to practical and ethical perspectives, since moderate and severe cases of ME/CFS may not be able to perform specific tests, *e.g.,* CPETs and tilt table testing, and the ethics of requiring patients to undertake a test likely to intensify pain and other symptoms could be questioned[154].

Assessing symptoms objectively, if possible, instead of using questionnaires and subjective measures, could also largely improve scientific progress. For example, the controversy about the claim that CBT and GET are effective interventions[49,288] without detrimental effects[289], which is challenged by others[50,290], could be resolved by subjecting the patients to objective tests, *e.g.,* CPETs and cognitive tests, before during and after CBT/GET. Especially since studies have shown that reduction in "fatigue" after behavioural interventions is not reflected by a clinical improvement in objective terms, *e.g.,* activity levels[291], distance walked in 6 min[49] or oxygen uptake[292]. Future trials into proposed effective pharmaceutical[52,53] and behavioural therapies[49,288] should be using objective measures to establish positive and negative effects in clear-defined patient populations impartially[293].

CONCLUSION

Although the labels ME and CFS are often used interchangeably, the diagnostic criteria for ME and CFS define two distinct, partially overlapping, clinical entities. ME, whether defined by the original[20] or the new consensus criteria[18], is not equivalent to CFS[19]. Muscle weakness, cognitive impairment, and above all, post-exertional "malaise", obligatory for the diagnosis ME, is not mandatory for the diagnosis CFS, while "chronic fatigue", the core feature of the diagnosis CFS, is not mandatory for ME.

Partly due to the subjective nature of the symptom-based definitions of ME and CFS and the use of self-report, questionnaires and subjective measures, some researchers and clinicians have questioned the physiological origin of the symptoms and qualified ME and CFS as functional somatic syndromes. The use of objective tests and measures to assess symptoms and functional limitations, *e.g.,* CPETs and cognitive tests, could resolve the controversy with regard to the nature of ME and CFS and the consequences for patients' lives and professional abilities. Looking at the medical, financial, social and emotional impact of ME and CFS on patients and society, and the future perspective of patients, an objective assessment of the symptoms and disability is a crucial step.

To explore the etiology and pathophysiology in well-defined ME and CFS patient subgroups research should employ objective test and biomarkers[1]. Therapies proposed to be effective for ME and/or CFS, should be evaluated by employing an objective assessment of the clinical status and biomarkers before, during and after the intervention in well-defined patient (sub)groups.

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REVIEW

Past, present and future of cyanide antagonism research: From the early remedies to the current therapies

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Abstract

This paper reviews milestones in antidotal therapies for cyanide (CN) spanning early remedies, current antidotal systems and research towards next generation therapies. CN has been a part of plant defense mechanisms for millions of years. It became industrially important in the nineteenth century with the advent of CN assisted gold mining and the use of CN as a pest control agent. The biochemical basis of CN poisoning was actively studied and key mechanisms were understood as early as 1929. These fundamental studies led to a variety of antidotes, including indirect CN binders that generate methemoglobin, direct CN binders such as hydroxocobalamin, and sulfur donors that convert CN to the less toxic thiocyanate. Research on blood gases at the end of the twentieth century shed new light on the role of nitric oxide (NO) in the body. The discovery of NO's ability to compete with CN for enzymatic binding sites provided a previously missed explanation for the rapid efficacy of NO generating antidotes such as the nitrites. Presently used CN therapies include: methemoglobin/NO generators (*e.g.*, sodium nitrite, amyl nitrite, and dimethyl aminophenol), sulfur donors (*e.g.*, sodium thiosulfate and glutathione), and direct binding agents [(*e.g.*, hydroxocobalamin and dicobalt salt of ethylenediaminetetraacetic acid (dicobalt edetate)]. A strong effort is being made to explore novel antidotal systems and to formulate them for rapid administration at the point of intoxication in mass casualty scenarios. New antidotes, formulations, and delivery systems are enhancing bioavailability and efficacy and hold promise for a new generation of improved CN countermeasures.

Key words: Cyanide; Hydrocyanic acid; Antagonist; Antidote; Cobinamide; Sulfanegen; Sulfane sulfur donor

Core tip: This paper reviews milestones in antidotal

therapies for cyanide (CN) spanning early history, current antidotal systems, and research towards next generation therapies. Presently used CN therapies include: methemoglobin/nitric oxide generators (*e.g.*, sodium nitrite, amyl nitrite, and dimethyl aminophenol), sulfur donors (*e.g.*, sodium thiosulfate and glutathione), and direct binding agents (*e.g.*, hydroxocobalamin and dicobalt edetate). New antidotes, formulations, and delivery systems are presently being developed for rapid administration at the point of intoxication in mass casualty scenarios. These hold promise for a new generation of improved CN countermeasures.

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INTRODUCTION

Cyanide (CN) refers to both the anion CN^- and the undissociated form of hydrogen cyanide (HCN). It is a weak acid with a pKa of 9.2, therefore at the body's pH it exists mainly in the HCN form. HCN can easily cross cellular, and subcellular membranes such as the blood brain barrier, and mitochondrial membranes.

CN is formed and is present in various spheres of Nature, such as the cosmos, volcanic eruptions, and lightning[1]. Certain plants, fungi, bacteria, and animals synthesize CN as a component of cyanogenic glycosides to provide a source of nitrogen and for self-defense[2,3]. Consuming cyanogenic plants (*e.g.,* cassava roots, yams, sorghum, maize) without proper preparation can lead to CN intoxication. The cyanogenic glycoside amygdalin is present in some pitted fruits like bitter almond, and apricot cherries[4]. Cyanogenic glycosides can eliminate HCN through a hydrolytic reaction[5]. Metabolism of other cyanogenic chemicals such as the cyanogen halides, and nitriles also leads to CN intoxication[6].

CN is present in smoke from fires (especially burning acrylonitrile, polyurethane, polyamide, wool, silk, rubber) and cigarettes[7-9]; in the vasodilating agent Nitroprusside (formulated as a CN complex)[10,11]; and in industrial settings where CN is widely used to complex metals - for example, CN is widely used in industry for gold extraction from ores[12].

CN has been used as a poison from antiquity. It was first isolated from cherry laurel[13]. Early CN poisonings were reported more than 200 years ago by Wepfer in 1679, and by Fontana in 1795[13], however, the first attempt to antagonize CN was reported later, in the 19th century[14]. Theories, based on biochemical mechanisms of CN antagonism were reported in the mid-20th century[15-20] and therapeutic agents became available for clinical application. After the recent recognition of CN as a military weapon and terror agent, intense research has led to a deeper understanding of CN biochemistry, and has fostered the development of novel CN countermeasures.

CN was used as a chemical weapon for the first time in World War Ⅰ. In World War Ⅱ the Nazi's used CN in gas chambers[21]. In the 1980's Iraq used CN against the Kurds[22]. CN was also involved in the Tokyo subway attack in 1995[23], in the first World Trade Center attack in 1993[24], and in the Chicago Tylenol disaster in 1982[25].

FROM RESEARCH TO THERAPY

Mechanism of cyanide toxicity/search for diagnostics

The primary biochemical basis of CN poisoning was known and published as early as 1929[26]. CN binds and inactivates several metal-containing enzymes, but the most important effect is attributed to the binding of Cyt c Oxidase, which is the terminal oxidase of the mitochondrial electron transport chain. This results in histotoxic hypoxia, due to the inhibition of cell oxygen utilization. As the result of suppressing the aerobic metabolic pathway, the less efficient anaerobic pathway becomes dominant, and pyruvate is reduced to lactic acid. The resultant acidosis leads to central nervous system (CNS) and myocardial depressions.

Higher CN doses (higher than 5XLD50) generate more complex responses[27]. For example, at higher doses, CN induces pulmonary arteriolar and coronary vasoconstriction that can result in cardiogenic shock/pulmonary edema[28], and stimulates chemoreceptors in the carotid artery and aorta resulting in hyper apnea. The antidotal effects of the vasodilators (*e.g.,* nitrites) and alpha-adrenergic blockers (*e.g.*, phenoxybenzamine) confirmed the vascular effects of CN, that are present along with the earlier discovered biochemical enzyme binding effects[29].

Lower doses of CN cause responses such as dizziness, headache, nausea, and vomiting as a result of inhibiting and interfering with the cellular enzymes. Chronic low dose CN exposure can result in Parkinson-like syndromes, confusion, and intellectual deterioration[30]. It is suggested that the wide-spread pathologic condition of tropic ataxic neuropathy is associated with the intense consumption of cassava with high CN-Glycoside content[31].

HCN absorbs rapidly through the mucus membranes and the skin. Inhaling HCN results in rapid intoxication, and if concentrations are high enough, death. When salts are ingested, the absorption from the GI tract is slower. About 60% of the absorbed CN binds to protein. It has high affinity to Cobalt and Fe^{3+}, but it also reacts with sulfur containing molecules in the body (see next paragraphs).

The diagnosis of CN intoxication is difficult in the absence of a direct assay for CN; however, cardiovascular and CNS depressions in smoke inhalation victims always suggest CN involvement. Generally,

symptoms, such as metabolic acidosis, coma, shock, seizures, bradycardia, are not specific, but if the patient is randomly collapsed, and not responding to oxygen treatment, CN exposure should be suspected. Laboratory tests can show saturated hemoglobin (Hgb) (oxygen is not utilized), lactic acidosis (blocked oxidative phosphorylation), and hyperglycemia (toxic effects on pancreatic beta-cells)[32]. CN caused death is confirmed chemically using a colorimetric test at the scene of the crime[33], followed by analysis by GC-MS[34]. Due to the relatively short half-life of CN in the blood[35], detecting major metabolites such as thiocyanate (SCN) and 2-aminothiazoline-4-carboxylic acid (ATCA) are recommended[36,37]. ATCA, as a stable CN metabolite has been established as an important forensic CN biomarker[38]. A recent review of the analysis of CN and its metabolites was published by Logue *et al*[39] in 2010.

Cyanide metabolism

Small amounts of CN can be metabolized in the body by various endogenous metabolic pathways, however, the rate of detoxification is slow (about 0.017 mg CN/body weight/min). The body can metabolize CN at even the lethal dose, if that is given slowly over the course of several hours[40]. In the most important endogenous detoxification reaction CN is converted to the less toxic SCN by reaction with thiosulfate and other endogenous sulfur donors in the presence of a sulfurtransferase enzyme, Rhodanese (Rh). Since Rh is a mitochondrial enzyme, and the inorganic thiosulfate has limited cell penetration capability, the serum albumin-sulfane complex plays a major role in ferrying sulfur donors to Rh[41]. Endogenous sulfur donors are formed from cysteine and methionine in the presence of sulfurtransferases such as Rh, Mercaptopyruvate Sulfur Transferase and Cystathionase. The second most important metabolic pathway is the formation of the chemically stable CN metabolite, ATCA, when CN reacts with endogenous cysteine[37]. Another important metabolic pathway is the formation of cyanocobalamin from hydroxocobalamin. These metabolic end-products are excreted by the urine, while unreacted CN is excreted in breath, urine and sweat. The most recent understanding of CN metabolism, the role of nitric oxide (NO) will be discussed in the next section.

Milestones in CN antagonism

At the end of the 19th and beginning of the 20th century scientists began a systematic search for ways of neutralizing the toxic effects of the deadly chemical. These investigations of CN antagonism prior to the 1930s resulted in the development of the classic CN therapies such as of sodium thiosulfate and sodium nitrite that are still in use worldwide. Table 1 lists some important therapies and the countries where they are presently employed in treating CN intoxication.

Methemoglobin formers: Historically the first CN poisoning remedy was the methemoglobin former amyl nitrite[42]. In studies on the biochemical mechanism of CN antagonism, it was found that while CN has low affinity to Hgb, it has high affinity to the oxidized form of Hgb, methemoglobin (MetHgb), resulting in the formation of the relatively stable cyano-MetHgb complex[15,16]. Amyl nitrite, the first MetHgb former used for CN antagonism, was followed subsequently by sodium nitrite. As early as 1933, the combination of the MetHgb formers amyl nitrite, sodium nitrite and sodium thiosulfate was reported to show significant enhancement in antidotal protection[15,16]. To overcome the disadvantage of the slow MetHgb formation by nitrites, a fast MetHgb former, 4-dimethylaminophenol (DMAP), was developed and investigated[43-45]. When DMAP was administered intravenously, CN was entrapped within the red blood cells with relatively high efficiency[46]. This fast acting MetHgb former was developed and used in Germany in the military and civilian population. It produced 2-3 x LD50 protection in a dog model when the MetHgb level was kept around 30%[47]. Similar CN antidotal protections were found with other MetHgb formers such as p-aminopropiophenone (PAPP), p-aminoheptanoylphenone, and hydroxylamine[48].

Sulfane sulfurs and sulfur donors as CN antidotes: As early as 1894 Lang[49] reported that sodium thiosulfate antagonizes CN intoxication by converting it to a biologically less active metabolite in rabbits. Pascheles[50] and Kahn[51] pointed out that in the liver enzymes enhanced the transformation of CN to SCN. During that time other endogenous organo sulfur molecules, such as cysteine, cystine and glutathione were also investigated as CN antidotes[52-54].

In parallel with the MetHgb binding theory reported by Chen *et al*[15] in 1933, Lang[55] reported the biotransformation reaction between CN and thiosulfate, in which thiosulfate served as a sulfur donor substrate for the mitochondrial sulfurtransferase, Rh. Investigating the substrate specificity of Rh, Sorbo reported that sulfur donors, such as aliphatic and aromatic thiosulfonates are superior sulfur donors than thiosulfate[56]. Rh (E.C. 2.8.1.1; thiosulfate:cyanide sulfurtransferase) was the first sulfurtransferase that was studied in detail[57,58]. The utilization of thiosulfate as CN antidote was reported to be limited by its short biological half-life, small volume of distribution, and limited ability to penetrate the mitochondrial membranes to reach the Rh enzyme[41]. Frankenberg *et al*[59] reported in 1975 that the presence of CN enhanced the cell penetration capability of thiosulfate, making thiosulfate more efficient as a therapeutic agent, rather than as a prophylactic agent. Sulfane sulfur molecules (containing multiple divalent sulfur atoms bound to each other) can serve as a sulfur donor to the Rh reaction. In 1981, Westley[60] reported that a series of sulfane sulfur compounds, such as thiosulfonates, polythionates persulfides, polysulfides, and elementary sulfur serve as substrates for the Rh reaction, however, the pharmacokinetic parameters and toxicity of some of these compounds limited their usage

Table 1 Present cyanide therapies worldwide

Antidotal therapy	Country availability
Sodium nitrite, amyl nitrite and sodium thiosulfate	Europe, Asia (Lilly kit; Talor kit; Pasadena kit) United States (Nithiodote™)
Hydroxocobalamin	European Union/United States (Cyanokit®)
4-Dimethylaminophenol	Germany/Austria/Netherlands
Dicobalt edetate	Netherlands/France/United Kingdom/Australia/Israel (Kelocyanor)

as CN antidotes.

The Westley lab reported that the reaction between CN and mercaptopyruvate (another type of sulfur donor), was catalyzed by 3-Mercaptopyruvate sulfurtransferase (E.C. 2.8.1.2) in the cytosol and mitochondria[61]. When mercaptopyruvate reacts with CN, both SCN and cyanohydrin are formed. Mercaptopyruvate enhances the antidotal effect of thiosulfate, or the thiosulfate + nitrite combination[19]. Westley *et al*[57] reported that serum albumin may also act as an endogenous sulfane sulfur donor, and can react with CN to form SCN.

Sodium thiosulfate and nitrite are the active components of the present CN antidotal combination therapy in the United States (Nithiodote™).

Cobalt compounds: Because cobalt has high affinity to CN, cobalt containing compounds can be used as CN antidotes. Hydroxocobalamin can react with CN by forming cyanocobalamin, which is excreted in the urine[62]. In a dog model, dicobalt edetate is superior to the classic nitrite + thiosulfate combination[63]. When cobalt chloride was administered in combination with thiosulfate or nitrite, it was reported that there was a striking enhancement between cobalt and thiosulfate, but not between the cobalt and the nitrite[64]. Due to its toxicity, cobalt chloride is not used as an antidote for humans[65].

Carbonyl compounds: CN is a nucleophile that reacts with carbonyl containing molecules, such as aldehydes and ketones, to form cyanohydrines. Sodium pyruvate was reported first to antagonize CN in a mice model[66]. The advantage of sodium pyruvate over the classic thiosulfate + nitrite antidotal combination is that it is actively transported intracellularly and can distribute to sites of CN localizations[67]. Sodium pyruvate has been reported to significantly enhance the protective effects of nitrite, but with thiosulfate this enhancement is negligible. However, by adding pyruvate to the combination of nitrite and thiosulfate, a striking protection was found[19]. The strong CN antidotal effects of alpha-ketoglutaric acid were reported as a result of cyanohydrine formation with CN[68].

Clorpromazine: Chlorpromazine was reported as a CN antidote as early as 1958[69]. The mechanism of its action is not known. It does not form MetHgb, bind CN, nor serve as sulfur donor, but it does enhance the antidotal effects of thiosulfate, and the thiosulfate + nitrite combination[70].

Oxygen/hyperbaric oxygen: The Way lab reported first the CN antidotal effect of Oxygen/Hyperbaric oxygen[71-73]. Oxygen alone does not protect against CN, but it does enhance the effects of thiosulfate and the thiosulfate + nitrite combination[74].

Way - classification of CN antidotes: Way[19] summarized the mechanisms of CN antagonism and classified the CN antagonists known at that time. For example, he described the four major steps involved in the antagonism of CN by nitrites and thiosulfate: (1) CN binds to Cytochrome C Oxidase to form CN-Cytochrome C Oxidase; (2) nitrite reacts with Hgb to form MetHgb; (3) CN binds to MetHgb to form Cyano-MetHgb; and (4) thiosulfate reacts with CN to form SCN. This mechanism explained how the classic CN therapy (thiosulfate + nitrite) works, how CN partitions between Cytochrome c Oxidase and MetHgb until it is eventually converted to SCN and eliminated from the body by the urine.

Way described four basic classes of CN antagonists: (1) class Ⅰ: Cyanide Binders: Class IA: MetHgb formers (such as nitrites and DMAP); Class IB: Cobalt compounds (such as dicobalt edetate and Hydroxocobalamin); Class IC: Carbonyl compounds (such as pyruvate); (2) class Ⅱ: Sulfur Donors: Class ⅡA: Thiosulfate; Class ⅡB: Thiosulfonates; Class ⅡC: Other Sulfur sulfanes; (3) class Ⅲ: Cyanide Binders and Sulfur Donors: such as mercaptopyruvate; and (4) class Ⅳ: Unknown Mechanism: Class ⅣA: Oxygen; Class ⅣB: Chlorpromazine.

New antidotal approach and new sulfur donor combination studies in the 20th century: The lower *in vivo* antidotal efficacy of thiosulfate relative to its *in vitro* efficacy highlighted how the limited cell penetration capability of thiosulfate adversely impacted its ability to reach the mitochondrial Rh[75]. Early investigations indicated the importance of externally administered Rh directly to the circulation to enhance the CN antidotal effect of thiosulfate[43,76-78]. However, when proteins (enzymes) are injected directly to the bloodstream, they are rapidly destroyed by proteolytic enzymes and the body's immune system. Therefore, the efficiency of this approach is limited[79]. To minimize the adverse immunologic reactions, a protective

environment is needed for the externally administered enzymes. The two major challenges of this approach of placing sulfur donor and Rh in a close proximity are: finding an appropriate sulfur donor with high sulfur donor reactivity, and developing an appropriate scheme for protecting Rh against macrophage recognition in the circulation[80]. Early investigations following this approach focused on Rh encapsulation within Carrier Erythrocytes[79-81]. To enhance the antidotal efficacy of the CN antidotal system of sulfur donor + externally administered Rh, organic thiosulfonates with superior sulfur donor reactivity were employed. When butane thiosulfonate was encapsulated with Rh in Carrier Erythrocytes, and administered in combination of sodium nitrite, a 14 x LD50 prophylactic protection was found[81]. To overcome the disadvantage of carrier erythrocytes (labor demanding encapsulation, prior blood typing), biodegradable, synthetic polymeric nano-delivery systems were employed[82].

The importance of this approach became suppressed when sulfur donors with higher lipophilicity, and higher cell penetration capability were employed. In 1999 Baskin *et al*[83] reported results on the *in vitro* and *in vivo* efficacy studies with various synthetic sulfur donors with different chemical structures and greater lipophilicity than thiosulfate. In 2006 Ashani *et al*[84] reported that garlic, and its main component, allicin, were beneficial in acute CN intoxications. Allicin breaks down spontaneously to form a variety of organosulfur molecules, such as diallyl-sulfide, diallyl-disulfide and diallyl trisulfide. In the presence of oil, allicin is transformed to ajoene and vinyl-dithiins[85,86]. Investigations of these specific garlic components proved that they are not superior to thiosulfate *in vitro* nor *in vivo,* even when they were applied with nano-intercalated Rh[82]. More recent investigations have examined naturally occurring sulfur donors from garlic and onion that have lower Rh dependence. These sulfur donors demonstrate superior sulfur donor reactivity to thiosulfate. As a result of these investigations, an advanced formulation and superior therapeutic antidotal protection from intramuscularly administered methylpropyl trisulfide was reported in a mice model[87]. Very recent investigations are focused on other garlic compounds as sulfur donors that can improve the ancient, but still clinically used thiosulfate + nitrite combination (Nithiodote™). Publications of the results of these investigations are presently in progress.

Mystery of nitrite/nitric oxide: Turning point in mechanism of CN antagonism: The traditional nitrite theory of MetHgb formation provided a simple explanation of the role of nitrites (sodium nitrites and amyl nitrites) in CN antagonism: Nitrites produce MetHgb; since MetHgb has higher affinity to CN than Cyt c oxidase, MetHgb removes CN from the binuclear heme center (Fea3-CuB) of Cyt c Oxidase, and the mitochondrial electron transport chain is able to return to its job of transferring electrons to oxygen and generating ATP. In this picture nitrites act as indirect CN scavengers[19,88]. When the nitrite + thiosulfate combination is applied, the CN that nitrite displaces reacts with thiosulfate to form SCN that is excreted in the urine[89,90]. However, additional research revealed that the blood MetHgb content needed to be around 15% to effectively antagonize CN[91]. However, when the recommended amyl nitrite dose is applied, only about 5%-7% of Hgb is oxidized to MetHgb, and at these low doses amyl nitrite still acts as an efficacious CN antidote.

The fact that rapid onset of antidotal efficacy by nitrites could not be explained by MetHgb formation suggested one or more additional therapeutic mechanisms. A turning point in understanding this mystery came when the mitochondrial NO synthase was discovered and the function of NO as a regulator in the electron transport chain was characterized[92].

NO regulates the conversion of oxygen to water by Cyt c Oxidase as follows: Ferri-heme a3 takes up electrons and is reduced to ferro-heme a3, NO enters its active site pocket before oxygen does, a nitrosyl-ferro-heme a3 derivative is formed that reacts with dioxygen. NO then dissociates from ferro-heme a3 (rate limiting step), and in the presence of an additional electron donor, nitrite is formed *via* an intermediate peroxynitrite. The cycle's last step is the conversion of peroxynitrite to nitrite and water. In this way, the cycle results in the reduction of oxygen by NO to form water and nitrite. When CN enters the mitochondria, it binds to Cyt c Oxidase. NO can alter the CN binding to Cyt c Oxydase and displace CN from the Cyt c Oxidase's binding site thus restoring its availability for Oxygen binding[93,94]. Pearce *et al*[95] 2003, suggested the reversal of CN inhibition of Cyt c Oxidase by NO occurs in the presence of excess reduced (ferro cyt c Oxidase) and oxygen. They followed the CN substitution by NO in the ferri-heme a3, through a 5-coordinate structure by electron paramagnetic resonance spectroscopy. They stated that NO does not simply act as a reversibly bound competitive inhibitor, but it is also an auxiliary substrate that is consumed and converted to readily releasable nitrite. The displaced CN may then be converted to SCN, or be scavenged by the circulating MetHgb. When external nitrite is added, it boosts the availability of the auxiliary substrate for the Cyt c Oxidase. Along with the NO and MetHgb mechanisms, the vasodilation effects of nitrites also contribute to the their antidotal effects against CN. These results by the Pearce *et al*[95] have provided strong evidence toward solving the puzzle of the mechanisms by which nitrite/NO antagonize CN.

The regulatory effect of NO on Cyt c Oxidase became a well-studied area[96,97]. The signaling molecule NO is generated endogenously from L-Arginine by nitric oxide synthase (NOS)[98]. In CN intoxication, due to the histotoxic hypoxia and lactic acidosis, the NOS activity is decreased, and an exogenous NO source is necessary to protect the cellular respiration. Exogenous nitrites can be transformed to NO even in the condition of

hypoxia[99,100].

Studies on isolated cells (where no Hgb/MetHgb) confirmed that NO inhibits CN by displacing it from Cyt c Oxidase[94]. When the NO donor S-nitroso-N-acetyl-DL-penicillamine was applied to the CN inhibited Cyt c Oxydase, CN was replaced by NO. However, when a NO scavenger 2-phenyl-4,4,5,5-teramethylimidazoline-1-oxy-3 oxide (PTIO) was present, the CN antagonism was blocked, providing strong experimental support for the role of NO as a CN displacement agent[96]. In 2010, Leavesley *et al*[101] used a selective NO scavenger, PTIO to explore CN inhibition by nitrites. In the presence of CN, both the PTIO consumption and the Cyt c Oxidase were inhibited. However, nitrite pretreatment reversed the CN inhibition of NO production and Cyt c Oxidase activity. Conversely, pretreatment with the NO scavenger PTIO negated the ability of the nitrites to antagonize CN. Cumulatively, these studies conclude that a key mechanism of CN antagonism by nitrites involves the generation of NO that competitively displaces CN from Cyt c Oxydase. The NO mechanism runs parallel to the MetHgb mechanism. The formation of each of these species, NO and MetHgb, plays an independent role in the *in vivo* antagonism of CN by nitrites.

Modern theory for old molecules (Nitrites): In 2013 Cambal *et al*[102] compared CN antidotal effects, and blood NO concentration when equimolar amounts of sodium nitrite and amyl nitrite were given to mice intraperitoneally or by inhalation after sub lethal CN doses. They reported that the toxic effects of iso-amyl alcohol formation from amyl nitrite, and the more efficient antidotal effects by sodium nitrite favored the use of sodium nitrite over iso-amyl nitrite. Agreeing with prior studies the authors noted that MetHgb formation was insufficiently rapid to explain the full antidotal effects of amyl nitrite[103]. They also noted that the sodium nitrite maintained efficacy even when MetHgb formation was suppressed. These studies suggested that the NO mechanism was primary and MetHgb formation secondary to the antidotal action of these nitrites. The authors also discussed the complexity of experimental interpretation when interferences due to anesthesia are present.

When the CN antidotal effects of the clinically used anti-angina drug isosorbide dinitrate (ISDN) were reported, NO formation was declared as the major mechanism of its CN antidotal efficacy. It was reported that ISDN has potential advantages over sodium nitrite because it is relatively non-toxic, and is less likely to form MetHgb[104].

Present therapies and recent research investigations in the United States

For treating CN poisoning, multiple antidotes exist and vary in regional availability: MetHgb generators (*e.g.,* sodium nitrite, amyl nitrite, and dimethyl aminophenol), sulfur donors (*e.g.,* sodium thiosulfate and glutathione), and direct binding agents (*e.g.,* hydroxocobalamin and dicobalt edetate). All currently marketed antidotes appear to be effective. In the United States, sodium nitrite and sodium thiosulfate and hydroxocobalamin are used as cyanide antidotes, while in France and several other European countries, only hydroxocobalamin is favored[105]. Antidotal mechanisms include chelation, formation of stable, less toxic complexes, MetHgb induction, and sulfane sulfur reaction with endogenous Rh enzyme. Research with the goal of finding new, safer and more effective cyanide antidotes continues[106].

Table 2 Novel cyanide therapies in development for rapid IM delivery in mass casualty situations

Novel therapy	Mechanism of action	Older analog
Cobinamide	Direct CN Scavenger	Hydroxocobalamin
Sulfanegen	CN Transformer (Leverages the mercaptopyruvate sulfur transferase enzyme)	Mercaptopyruvate
Next generation sulfane sulfur donors	CN Transformer (Leverages the rhodanese enzyme)	Thiosulfate

CN: Cyanide.

The two currently FDA approved CN countermeasures, Nithiodote™ (sodium nitrite and sodium thiosulfate) and Cyanokit® (hydroxocobalamin), each have limitations [*e.g.,* sodium nitrite: intravenous (IV) administration, hypotension, methemoglobinemia; thiosulfate: IV administration, slow onset of action; hydroxocobalamin: IV administration, large volume]. Furthermore, the common requirement for IV administration renders broad use of these CN countermeasures unrealistic in a mass CN exposure event. The distinct need remains to develop a CN countermeasure suitable for mass casualties. Recent investigation efforts focus on developing efficient, easy to administer (*e.g.,* intramuscular) CN countermeasures, which may also be used in combination with other new generation countermeasures. Table 2 shows the classification of recent CN antidotes under development in the United States related to the two present CN therapies of Cyanokit® and Nithiodote™.

Hydroxocobalamin

Hydroxocobalamin is a vitamin B derivative known as vitamin B_{12a}. The compound is a hygroscopic, odorless, dark red crystalline powder which is freely soluble in water and ethanol, and practically insoluble in acetone and diethyl ether. It is the hydroxylated active form of vitamin B_{12} differing in that hydroxocobalamin has a hydroxo moiety linked to a cobalt ion while cyanocobalamin, known as vitamin B_{12} has a cyano moiety. The latter is used to treat pernicious anemia while hydroxocobalamin, based on its strong cyanide binding ability is a potent antidote against cyanide. Hydroxocobalamin has a large molecular weight and a trivalent

cobalt ion that is coordinated by a tetrapyrrol ring. Its mode of action is attributed to its ability to form cyanocobalamin through binding cyanide ion by substituting the aforementioned hydroxo ligand[107,108].

Its pharmacokinetic properties can be characterized by significant plasma protein binding and the formation of various cobalamin-(Ⅲ) complexes after intravenous administration. Free and total cobalamin-(Ⅲ) complexes have half-lives of 26-31 h and overall urinary excretion accounts for 60%-70% of the administered dose[108].

The first reports of its antidotal efficacy were documented in 1952 when an experimental cyanide poisoning of mice was conducted. Ever since, hydroxocobalamin has proved its efficacy in combating cyanide intoxication both in animal models and humans. Mice, rats, guinea pigs, beagle dogs and Yorkshire pigs are some of the species that were included in hydroxocobalamin studies. Alongside the large variety of species included in the investigations various administration methods, such as intravenous, intraperitoneal and intracerebral micro dialysis were also tested. The studies also included pre- and post-poisoning set-ups and cyanide intoxications of various natures including inhalation and parenteral[109-114].

The efficacy of hydroxocobalamin was also seen in human poisonings. In these cases the antidotal effect of the compound, although originally developed as a monotherapy was seen either alone or in combination with other agents, such as 100% oxygen, sodium nitrite and sodium thiosulfate. The therapy was applied in cyanide poisonings originating from various sources including ingestion (*e.g.,* cyanide salts and hydrogen cyanide) and inhalation (*e.g.,* smoke). Survival rate depended on many factors but most notably hydroxocobalamin therapy was especially helpful when administered before anoxic brain damage occurred due to the cardiac arrest of the patients[114-116].

Hydroxocobalamin is available as an United States Food and Drug Administration (FDA) and European Medicines Agency (EMA) approved formulation. The development of the medication was initiated in the 1980s based on request from the Fire Brigade of Paris and antipoisoning center of Paris. The product was approved in France in 1996 and approved in the United States fast track in 2006, followed by EMA approval in 2007. The antidotal kit comprises two glass vials. Each vial contains 2.5 g of hydroxocobalamin in the form of lyophilized powder, a transfer spike for reconstitution, and an intravenous tubing system. Prior to administration the product is reconstituted with 100 mL of preferably 0.9% sodium chloride solution for injection, or lactated Ringer or 5% glucose solution for injection. The mixture is shaken and then administered intravenously over a period of 7.5 min. Hydroxocobalamin has shown itself to be an effective CN antidote *via* intravenous administration. In the pivotal studies which due to ethical reasons were not controlled studies but included patients with suspected or known cyanide-poisoning a survival rate of 58% was seen[108,117].

Cobinamide

Cobinamide is the penultimate precursor in the biosynthesis of cobalamin, lacking the dimethylbenzimidazole nucleotide tail coordinated to the cobalt atom in the lower axial position.

Thus, whereas cobalamin has only an upper ligand binding site, cobinamide has two - both an upper and a lower - ligand binding sites. Moreover, the dimethylbenzimidazole group has a negative trans effect on the upper binding site, thereby reducing cobalamin's affinity for ligands. As a result of these cobinamide has a higher affinity with an overall K_a of 10^{22} mol/L for cyanide ion (binding affinity for first cyanide ion is 10^{14} mol/L and for second ion is 10^{8} mol/L) than cobalamin; for the sake of comparison K_a is 10^{12} mol/L for cobalamin. On the basis of K_a values cobinamide should be a more effective cyanide detoxifying agent than cobalamin[118]. Furthermore, from the aspect of antidote formulation an advantageous feature of cobinamide is that it is at least five times more water soluble (as aquohydroxocobinamide) than hydroxocobalamin[119].

As *in vitro* affinities are not always in line with the *in vivo* conditions (*e.g.,* intracellular proteins may bind cobinamide and cobalamin to varying degrees), many *in vivo* investigations on cell lines and animal models were carried out to examine the cyanide detoxifying properties of cobinamide and to compare it with cobalamin and other antidotes.

The ability of equimolar doses of cobinamide and hydroxocobalamin to reverse the effects of cyanide exposure in New Zealand white rabbits (n = 16) was investigated by Brenner *et al*[119]. CN toxicity was induced by intravenous infusion and the animals were monitored continuously noninvasively by diffuse optical spectroscopy.

Rabbits were divided into three groups: controls (n = 5) received intravenous saline, 6 rabbits were treated with intravenous hydroxocobalamin, and 5 rabbits with intravenous cobinamide following CN exposure. At the end of the sodium cyanide (NaCN) infusion (10 mg in 60 cc normal saline for 60 min), 5 cc of normal saline or 0.0816 millimoles of cobinamide or hydroxocobalamin dissolved in 5 cc of normal saline were infused over 30 s. Cobinamide caused significantly faster and more complete recovery of oxy- and deoxyhemoglobin concentrations in CN-exposed animals over hydroxocobalamin with a recovery time constant of 13.8 ± 7.1 min.

Broderick *et al*[118] found cobinamide to be several-fold more effective than cobalamin in reversing CN inhibition of oxidative phosphorylation in mammalian cells; in rescuing mammalian cells and Drosophila melanogaster from cyanide toxicity; and in reducing CN inhibition of Drosophila Malpighian tubule secretion. Cobinamide was as effective when administered up to 5 min post-CN exposure as when used pre-exposure for prophylaxis[118].

Currently marketed antidotal formulations for CN poisoning must be given by IV administration, limiting their use in treating mass casualties. It is advantageous that cobinamide could be delivered by other routes, *e.g.,* oral ingestion, inhalation, too[118,120].

Cobinamide was studied in both an inhaled and intraperitoneal model of CN poisoning in mice by Chan *et al*[120]. They found cobinamide more effective than hydroxocobalamin, sodium thiosulfate, sodium nitrite, and the combination of sodium thiosulfate-sodium nitrite. Compared to hydroxocobalamin, cobinamide was 3 and 11 times more potent in the intraperitoneal and inhalation models, respectively.

The use of intramuscular cobinamide sulfite to reverse CN toxicity-induced physiologic changes in a sub lethal CN exposure animal model was investigated by Brenner *et al*[121]. New Zealand white rabbits (n = 11) were given 10 mg sodium cyanide intravenously over 60 min. To follow the effect of antidote, tissue oxy- and deoxyhemoglobin concentrations were monitored using quantitative diffuse optical spectroscopy and continuous-wave near-infrared spectroscopy. After completion of the CN infusion, the rabbits were treated intramuscularly with cobinamide sulfite (n = 6) or vehicle (controls, n = 5). Intramuscular administration led to rapid absorption of cobinamide and the molecule was extremely effective at reversing the physiologic effects of CN. Recovery time to 63% of their baseline values in the central nervous system occurred within a mean of 1032 min in the control group and 9 min in the cobinamide group, with a difference of 1023 min. In muscle tissue, recovery times were 76 and 24 min, with a difference of 52 min[121].

When hydroxocobalamin and cobinamide were compared on a nonventillated swine model it was reported that both hydroxocobalamin and cobinamide rescued severely CN-poisoned swine from apnea, however, the dose of cobinamide was one fifth that of hydroxocobalamin to get the same protection[122].

In another study in which cobinamide sulfite was administered *via* intramuscular injection, cobinamide was rapidly absorbed. Mice recovered from a lethal dose of CN even when cobinimide was injected after they had been apneic for over 2 min. Cobinamide sulfite at doses up to 2000 mg/kg exhibited no clinical toxicity[120].

Broderick *et al*[123] observed that cobinamide is highly effective in neutralizing CN ions released by nitroprusside in cultured mammalian cells, Drosophila melanogaster, and mice. Sodium nitroprusside is used to treat hypertensive emergencies and acute heart failure. It acts by releasing NO, a highly potent vasodilator, but for each NO molecule released, five cyanide ions are released, too; thus limiting the safe use of this therapy. To avoid this side effect a CN scavenger could be beneficial when administering nitroprusside. It was reported that cobinamide could neutralize nitroprusside-released CN without having any effect on nitroprusside-released NO, thus it could be a valuable adjunct to nitroprusside therapy[123].

It is worth mentioning that in addition to cyanide, cobinamide is capable of reacting with NO. However, the binding constant of cobinamide is substantially lower for NO than it is for cyanide ($\approx 10^{22}$ mol/L for cyanide; $\approx 10^{10}$ mol/L for NO)[124]. On this basis when given in excess of available CN, cobinamide may potentially induce vasoconstriction and increase blood pressure, as has also been observed with hydroxocobalamin[125].

It can be concluded that cobinamide and its sulfite-salt are effective cyanide detoxifying agents that have the potential to serve as CN antidotes for smoke inhalation victims and persons exposed to CN used as a weapon of mass destruction.

Sulfanegen

Sulfanegen is the water-soluble prodrug of 3-mercaptopyruvate that was developed in the early 1990's to overcome the problem of the relatively low serum stability of the sulfur donor 3-mercaptopyruvate[126]. Sulfanegen is a dimer that dissociates non-enzymatically in physiological systems to two equivalents of the monomer 3-mercaptopyruvate. The endogenous enzyme 3-mercaptopyruvate sulfur transferase (3-MPST) catalyzes the transfer of reactive sulfane sulfur from 3-mercaptopyruvate to CN resulting in the formation of thiocyanate and pyruvate[127,128]. Compared to Rh, the 3-MPST enzyme is available in both the mitochondria and cytoplasm, whereas rhodanese is present only in the mitochondria of hepatic and renal tissues. It was shown that a high amount of 3-MPST exists in the tissue of the brain, specifically in the cerebellum. This strong presence of detoxifying enzyme in the brain holds therapeutic promise, because much of the damage from CN intoxication is localized in the heart and brain.

The other potential advantage with sulfanegen is the fact that it exerts its effects in less than 3 min. With hydroxocobalamin, a fifteen-minute intravenous infusion is required to deliver a standard dose. The discovery of the highly water-soluble sulfanegen triethanolamine for development as an intramuscular injectable antidote was reported by Patterson *et al*[129]. The potential of intramuscular and intravenous sulfanegen sodium treatment to reverse CN effects was evaluated in a rabbit model (n = 35). Changes associated with CN exposure and reversal were monitored by diffuse optical spectroscopy and continuous wave near infrared spectroscopy. Sulfanegen sodium was shown to reverse the effects of CN exposure on oxy- and deoxyHgb rapidly, significantly faster than in case of control animals. Red blood cell CN levels also returned to normal levels faster with both intramuscular and intravenous sulfanegen sodium treatment than with control treatments[126].

Severe CN toxicity - occurrence of severe lactic acidosis accompanied by significant elevation in blood CN levels - was induced in juvenile pig models to demonstrate the CN antagonism capability of sulfanegen[127]. Anesthetized pigs (n = 8) received a high-dose intravenous infusion of sodium nitroprusside SNP (100 mg/h) for 2 h to induce CN toxicity. Then, four

pigs received 3 doses of sulfanegen sodium (2.5 g *i.v.*) and four pigs received placebos. Administration of the sulfanegen antidote resulted in progressively significant reduction in blood lactate and CN levels with 100% survival ($P < 0.05$), whereas the placebo-treated pigs deteriorated and did not survive ($P < 0.05$). In another group of pigs ($n = 6$) severe CN toxicity was induced by NaCN and at peak toxicity (value determined during preliminary measurements), the animals were given sodium sulfanegen (2.5 g *i.v.*) followed by a repeat dose 60 min later in surviving animals. Without sulfanegen the NaCN injection used in this study resulted in CN toxicity, accompanied by severe lactic acidosis, and mortality in all the pigs. Sodium sulfanegen reversed NaCN-toxicity and prevented mortality in all the pigs treated with this antidote[127].

The combination of cobinamide and sulfanegen was explored by Chan *et al*[120] using a non-lethal and two different lethal models - a CN injection and a CN inhalation - of CN poisoning in mice. The effect of the two antidotes was found to be at least additive when used together in all the models used in this study. At doses where all animals died with either drug alone, the combination yielded 80% and 40% survival in the injection and inhalation models, respectively. Similarly, drug doses that yielded only 40% survival with either drug alone, yielded 80% and 100% survival with the combination therapy in the injection and inhalation models, respectively[120].

New sulfur donors

As indicated earlier, very recent investigations in the Petrikovics lab have focused on naturally occurring sulfur donors with lower Rh dependence (scavenger type mechanism), that have superior sulfur donor reactivity to thiosulfate. These sulfur donors were first extracted from garlic compounds and show great promise as successors to the ancient antidote thiosulfate, which is still clinically used in the thiosulfate + nitrite combination (Nithiodote™). Publications of the results of these investigations are presently in progress.

CONCLUSION

Table 1 shows the recent therapies in the US and some European countries, and Table 2 indicates the relations of the new generation CN antidotes to the recent CN therapies in the US. The roots of the present clinically employed therapies reach back to research efforts initiated in the early 20th century. CN intoxication from suicides, homicides, fires, industrial accidents, and potential terrorist attacks, presents a tremendous need for new, rapidly acting and efficient tools to antagonize/treat the toxic and lethal effects of CN in both isolated and mass casualty scenarios.

The lead new generation CN countermeasure cobinamide is a successor of the ancient, but still employed therapy of hydroxocobalamin. Sulfanegen is a next generation successor of mercaptopyruvate. The new generation of sulfane sulfur donors are successors of the classic sulfur donor sodium thiosulfate. These novel sulfane sulfur donors are organo-sulfur molecules that were originally found in garlic and onion. They have other potentially positive health effects[130] and work efficiently against CN intoxication without requiring the catalytic mediation of sulfurtransferases.

New combinations, formulations and nano-delivery systems of these next generation antidotes with enhanced bioavailability, and efficacy, provide strong hope for the gradual replacement of present therapies with improved new drugs and therapies for CN intervention.

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Current *Helicobacter pylori* treatment in 2014

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Abstract

Helicobacter pylori is one of the most commonly seen bacterium worldwide. It's in the etiology of multiple gastrointestinal diseases, ranging from gastritis to gastric carcinoma. The antimicrobial therapies, which are frequently prescribed empirically, are losing their effectivity as a result of the increasing antimicrobial resistance. As the standard triple therapy is now left especially in areas with high-clarithromycin resistance due to decreased eradication rates, quadruple therapies are recommended in most regions of the world. Alternatively, concomitant, sequential and hybrid therapies are used. There is still a debate going on about the use of levofloxacin-based therapy in order to prevent the increase in quinolone resistance. If no regimen can achieve the desired eradication rate, culture-guided individualized therapies are highly recommended. Probiotics, statins and n-acetylcysteine are helpful as adjuvant therapies in order to increase the effectiveness of the eradication therapy. Herein, we focused on different eradication regimens in order to highlight the current *Helicobacter pylori* treatment.

Key words: *Helicobacter pylori*; Eradication; Treatment; Bismuth-quadruple therapy; Sequential therapy; Concomitant therapy; Hybrid therapy

Core tip: In this review, we focused on different treatment regimens used for *Helicobacter pylori* eradication. The worldwide increase in antibiotic resistance, especially clarithromycin, caused change in the preferred initial treatments. The efficiency of bismuth-quadruple therapy, sequential, concomitant and hybrid therapies are emphasized in relation to each other. In addition, adjuvant therapies to increase the efficiency are reviewed. In conclusion, the optimal approach for eradication was found to be the individualized therapy.

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INTRODUCTION

Helicobacter pylori (*H. pylori*), a gram-negative

Table 1 Regimens for *Helicobacter pylori* treatment

Standard triple therapy (7-14 d)	PPI - standard dose, *bid* Clarithromycin - 500 mg, *bid* Amoxicillin - 1 g, *bid*
Bismuth quadruple Therapy (10-14 d)	PPI - standard dose, *bid* Bismuth - standard dose, *qid* Tetracycline - 500 mg, *qid* Metronidazole - 500 mg, *tid*
Sequential therapy (5-d dual therapy followed by a 5-d triple therapy)	Dual therapy; PPI - standard dose, *bid* Amoxicillin - 1 g, *bid* Triple therapy; PPI - standard dose, *bid* Clarithromycin - 500 mg, *bid* Metronidazole - 500 mg, *bid*
Concomitant therapy (7-10 d)	PPI - standard dose, *bid* Clarithromycin - 500 mg, *bid* Amoxicillin - 1 g, *bid* Metronidazole - 500 mg, *bid*
Hybrid therapy (7-d dual therapy followed by a 7-d quadruple therapy)	Dual therapy; PPI - standard dose, *bid* Amoxicillin - 1 g, *bid* Triple therapy; PPI - standard dose, *bid* Amoxicillin - 1 g, *bid* Clarithromycin - 500 mg, *bid* Metronidazole - 500 mg, *bid*
Levofloxacin-based triple therapy (10-d)	PPI - standard dose, *bid* Levofloxacin - 500 mg, *qd* Amoxicillin - 1 g, *bid*
Rifabutin-based triple therapy (7-14 d)	PPI - standard dose, *bid* Amoxicillin - 1 g, *bid* Rifabutin - 150 mg, *qd*
Culture-guided therapy (10-d)	PPI - standard dose, *bid* Bismuth - standard dose, *qid* Two antibiotics selected by antimicrobial sensitivity tests

PPI: Proton pump inhibitor; *bid*: Twice daily; *qd*: Once daily; *qid*: Four times daily; *tid*: Three times daily.

microaerophilic spiral bacillus discovered in 1983, affects nearly 50% of the world's population. While the prevalence is as high as 90% in developing countries, it is below 40% in developed countries, besides Japan[1]. Many studies have revealed a strong relation between the organism infection and gastric disorders, especially functional dyspepsia, peptic ulcer disease, gastric carcinoma and mucosa associated lymphoid tissue-lymphoma[2]. Moreover, extra-digestive diseases are also associated with *H. pylori*; idiopathic thrombocytopenic purpura and idiopathic iron deficiency anemia[3]. Therefore, eradication of *H. pylori* is an important issue, which still remains unsolved. Today, there is still not a single optimal antibiotic treatment for eradication. Herein, we focused on many articles published over the past years on *H. pylori* eradication regimens and their efficacy.

FIRST-LINE THERAPY

In the 90's, Bazzoli *et al*[4] first proposed the clarithromycin based standard triple therapy -clarithromycin, proton pump inhibitor (PPI) plus amoxicillin or metronidazole given for 7-14 d - which then became the gold standard in the treatment of *H. pylori* (Table 1). While the high eradication success (> 80%), optimal safety profile and relative simplicity made this regimen one of the standard of care treatments for first-line eradication of *H. pylori,* the raise in clarithromycin resistance in the 2000's caused a significant decline in the efficacy of this standard regimen[5]. In their study, Lee *et al*[6] reported the factors causing treatment failure as; age ⩾ 50 years, female gender, body mass index < 25 kg/m^2, amoxicillin, and/or clarithromycin resistance by univariate analysis. On the other hand, clarithromycin resistance was the only worthy parameter found by multivariate analysis. Clarithromycin works by interrupting the bacterial protein synthesis and resistance is caused by a mutation in the organism, which prevents the binding of the antibiotic to the ribosome of *H. pylori*[7]. The use of clarithromycin for respiratory and gastrointestinal infections causes the increased resistance rates[8]. High bacterial load, strain types, high gastric acidity and low compliance are the other contributors to eradication failure[9]. New evidence suggests that treatment failure may be due to the ability of the bacterium to control T-cell responses[10]. The clarithromycin resistance rate is variable in different parts of the world; the most recent report from European Helicobacter Study Group stated the primary resistance rate for clarithromycin as 17.5%[11]. The threshold of 15%-20% prevalence is used to classify low or high clarithromycin resistance[12]. That determines the approach to *H. pylori* eradication.

First-line treatment may be split into two groups; treatment in areas with low clarithromycin resistance and in areas with high clarithromycin resistance. The most frequently used regimen in areas with low clarithromycin resistance is standard triple therapy while bismuth-containing quadruple therapy is also an alternative. The duration of therapy is suggested as 14 d by meta-analyses with eradication rates 5% higher than those with 7 d[13]. A Cochrane systemic review looked at 75 eligible studies and found that eradication rate was 83.5% for PPI, amoxicillin and clarithromycin; for PPI, clarithromycin and metronidazole the rate was 68.6% and for PPI, amoxicillin and metronidazole it was found to be 82%; each therapy lasting 14 d[14].

Increased dose of PPI, as strong suppression of acid secretion is essential for the stability and biological activity of antibiotics, or the increased length of treatment are factors improving the efficacy rates. A meta-analysis showed a greater beneficial effect with a double dose of esomeprazole while another meta-analysis showed lower cure rates in hosts who are extensive PPI metabolizers (depending on their cytochrome P450 status which PPI function relies on)[13]. Taking this into account, a study found out that administration of a PPI four times daily with amoxicillin or metronidazole in clarithromycin resistance, may result with eradication rate of 98%[15]. In a study done by Altintas *et al*[16] comparing the efficacy of different proton pump inhibitors - omeprazole, lansoprazole and pantoprazole - in combination with amoxicillin and clarithromycin in the first line eradication of *H. pylori*, there wasn't any difference between the three groups[16]. Another meta-analysis done by Vergara

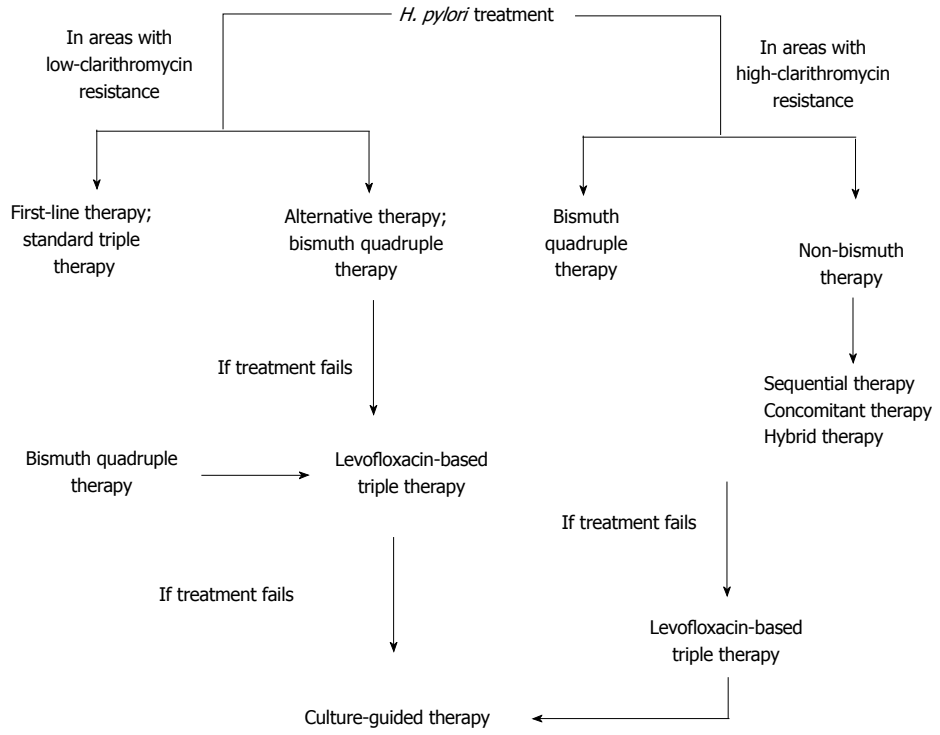

Figure 1 ***Helicobacter pylori*** **treatment algorithm.**

et al[17] including fourteen studies found no difference between proton-pump inhibitors when used in standard triple therapy for *H. pylori* eradication. The *in vitro* antibacterial activity of proton pump inhibitors vary but still the eradication rates are similar which suggests that acid inhibition is the main antibacterial mechanism of proton-pump inhibitors *in vivo*[17]. On the other hand, although the eradication rate of triple therapy with clarithromycin fell to 65%, it remained as high as 84% with metronidazole[18]. It is mostly due to the fact that metronidazole resistance may be overcome by increasing the dose and prolonging treatment duration[19]. In areas with high clarithromycin resistance (*i.e.*, Spain, Turkey, Alaska, China and Japan) bismuth quadruple, sequential, concomitant and hybrid therapies may be used[20].

BISMUTH QUADRUPLE THERAPY

Bismuth quadruple therapy is recommended as a first-line therapy in areas with high clarithromycin resistance, as an alternative first-line therapy in areas with low clarithromycin resistance or as an empirical treatment when clarithromycin therapy fails (Figure 1)[21]. It involves combination of a PPI, bismuth subsalicylate, metronidazole and tetracycline for 10 to 14 d (Table 1). This treatment is ideal as a second-line therapy, as recommended by The Maastricht Ⅳ Consensus Report[22] and the Second Asia-Pacific Consensus Guidelines[23]. Since it doesn't contain clarithromycin, compliance with the regimen is high and also metronidazole resistance *in vitro* does not affect the outcome significantly[19]. Salazar *et al*[24] reported an eradication rate greater than 95% with 14-d bismuth-quadruple therapy. A study from China, looking at the efficacy of bismuth quadruple therapy with lansoprazole as PPI and tetracycline/amoxicillin with metronidazole or furazolidone yielded eradication rates of 87.9%-95.2%; best outcome being the combination of lansoprazole, bismuth, amoxicillin and furazolidone[25].

Among the meta-analyses comparing standard triple therapy with bismuth quadruple therapy as first-line treatment, a study done by Venerito *et al*[26] showed eradication rate of 77.6% with bismuth quadruple therapy whereas it was 68.9% with clarithromycin-based standard therapy. A meta-analysis done by Luther *et al*[27] concluded that quadruple and triple therapies yielded similar eradication rates when applied as primary therapy for *H. pylori* infection and revealed similar side effects. In regions with high clarithromycin resistance, it is suggested as first-line therapy and achieved eradication rate of 82% compared to standard triple therapy[12]. Marin *et al*[28] reported eradication rates of 76%, 77% and 82% for 7, 10 and 14 d, respectively, with bismuth quadruple therapy when they applied bismuth quadruple therapy as rescue therapy. Also, Liang *et al*[25] declared eradication rates of > 90% in patients who did not respond to previous therapies, including those with metronidazole resistance.

As bismuth is concentrated in *H. pylori* and the organism doesn't develop resistance to it, applying bismuth quadruple therapy is advantageous over non-bismuth therapy[7]. The main limitations of this therapy are non-availability of bismuth salts or tetracycline in some countries as well as potential toxicity of bismuth. Nevertheless, no differences in terms of tolerability

were found between non-bismuth and bismuth containing therapies in a study among 4763 patients, except dark stools being more common in bismuth-containing group[29]. A single capsule formulation has been developed (Pylera) to overcome the complexity of quadruple therapy and showed good efficacy[20]. If the bismuth quadruple therapy is not available, sequential, concomitant or hybrid therapies may be administered.

SEQUENTIAL THERAPY

Sequential therapy, proposed by a group of Italian researchers, is a novel treatment intending to administer the antimicrobials in sequence. It is a 10-d treatment consisting of 5 d of PPI therapy with amoxicillin followed by a further 5 d of PPI with clarithromycin and metronidazole (Table 1). The main goal of sequential therapy, which has shown to have success rate of 90%-94% in several studies, is to overcome clarithromycin resistance[20]. It has been deemed that administration of amoxicillin deteriorates the bacterial cell wall, which ends up transferring clarithromycin out of the bacteria by preventing the development of efflux channels[5]. A study from China showed that sequential therapy achieves significantly higher eradication rates than triple therapy in patients with clarithromycin-resistant strains; while neither treatment was good enough to reach an eradication rate higher than 55% when clarithromycin and metronidazole resistance exists[30]. Similar results were obtained in a study from Korea where a high prevalence of clarithromycin resistant *H. pylori* is seen; eradication rates of 79% and 62% with sequential and triple therapy, respectively[31]. One study from Turkey, where clarithromycin resistance is high, reported success rate of 78% with sequential therapy *vs* 53% with standard triple therapy[32]. However, a recent meta-analysis evaluating *H. pylori* eradication rates in children showed that although sequential therapy is superior to 7-d triple therapy, it is not significantly better than 10- or 14-d triple therapy[13]. Three meta-analyses that took place in Asia in 2014 comparing efficacy of sequential therapy with standard triple therapy, favored sequential therapy over standard therapy[33-35]. Also, in a meta-analysis done by Zullo *et al*[36] success rate of sequential regimen was found higher (approximately 10%) compared to the triple therapy. Beside the mentioned study and meta-analyses, in Latin America countries, studies showed that standard triple therapy for 14 d was superior to sequential therapy (82% *vs* 76.5% respectively). This conflicting data might be due to variations in the prevalence of antibiotic resistance, which are lower in Latin America countries[37].

Sequential therapy is also recommended as first-line therapy like bismuth quadruple therapy in areas with high-clarithromycin resistance, *i.e.*, Italy and China with 90%-92% success rates[20]. In areas where bismuth drugs are not available, it may be necessary to prescribe sequential therapy[22]. A study done by Liu *et al*[38] reported that 10-d sequential and modified bismuth quadruple therapies are both highly effective as empirical first-line therapies in Chinese patients.

CONCOMITANT THERAPY

Concomitant therapy is proposed in order to reduce the complexity of sequential therapy which involves simultaneous administration of three antibiotics and a PPI for 10 d (Table 1). This treatment is used in areas where high-clarithromycin resistance is present and bismuth-based quadruple therapy is not available (Figure 1). When compared with standard triple therapy in meta-analyses of randomized trials concomitant therapy was found to be superior to standard triple therapy with 90% eradication rate[39]. In the presence of dual-resistance (100% clarithromycin and 91% metronidazole resistance), eradication rate was only 55% with concomitant therapy[5]. However, in a recent controlled trial done in Greece where clarithromycin resistance was 25% and metronidazole resistance was 40%, eradication rate was 90% under concomitant therapy[40]. Considering the sequential therapy, the eradication rates were found similar in a prospective randomized clinical trial in Spain; 91% with concomitant therapy and 86% with sequential therapy[41]. In addition, Gatta *et al*[42] found no superiority between sequential and concomitant therapies. If the concomitant therapy fails, empirical therapy becomes difficult due to exposure to both metronidazole and clarithromycin; as a result, a levofloxacin-containing or rifabutin-containing regimen may be necessary[7].

HYBRID THERAPY

Hybrid (dual-concomitant) therapy, which consists of two steps; 7 d of PPI and amoxicillin followed by a PPI and clarithromycin, amoxicillin and metronidazole, intends to overcome resistance with the benefits of four drugs of the concomitant therapy (Table 1). This therapy was first described by Hsu *et al*[43] and eradication rate of 99% by per-protocol and 97.4% by intention-to-treat analysis were obtained in 117 treated patients. Studies done involving Spanish and Italian people showed similar eradication rates (approximately 90%) for both hybrid and concomitant therapies[42]. Comparing hybrid and sequential therapies, 89.5% and 76.7% success rates were reported in a study with similar severe adverse effects[13]. More studies are needed to understand the efficacy of hybrid therapy.

LEVOFLOXACIN/RIFABUTIN BASED QUADRAPLE THERAPY

Levofloxacin-based therapy is recommended whenever bismuth quadruple therapy fails, in areas with both low and high clarithromycin resistance. It is a 10-d treatment with amoxicillin, levofloxacin and PPI (Table 1). Although eradication rate is around 90% when used

instead of clarithromycin in either triple or sequential therapies, the obstacle to use levofloxacin as a first-line treatment is the increasing frequency of quinolone resistance; which is currently 40% in America, 20% in Europe and 10% in Asia[5,44]. The meta-analysis done among patients who failed eradication with standard triple therapy showed better eradication rates with levofloxacin triple therapy than bismuth quadruple therapy, 81% and 70% respectively[45]. Including levofloxacin instead of clarithromycin in sequential therapy showed higher eradication rates in a study done by Gatta *et al*[42] Moxifloxacin and sitafloxacin may also be used but there is no evidence supporting advantage over levofloxacin. It is better to reserve fluoroquinolones for use in rescue regimens when the therapy with clarithromycin or metronidazole treatment fails.

Another salvage therapy is with rifabutin (Table 1). The advantage is the low frequency of rifabutin resistance. In a study done in Korea, where high prevalence of levofloxacin resistance is seen, amongst patients who had failed two initial regimens rifabutin triple therapy had better eradication rates than levofloxacin triple therapy, 71% and 57% respectively[46]. 50% success rate was achieved by rifabutin-based therapy in patients who had failed clarithromycin-, metronidazole- and levofloxacin-based therapy[47].

THIRD-LINE THERAPY

The Maastricht Ⅳ Consensus Report recommends antimicrobial susceptibility testing when the second-line treatment fails[22]. Although it will provide the best choice of antibiotics that can be used, the sensitivity of culture has been reported as < 60%[18]. In a study done by Cammarota *et al*[48], 90% of eradication rate was obtained among patients treated with a culture-guided third-line regimen. Culture-guided therapy is a 10-d quadruple therapy comprising a PPI, bismuth and two antibiotics selected by antimicrobial sensitivity tests (Table 1).

ADJUVANT THERAPIES

There are multiple approaches identified to overcome the side effects and increase the efficacy of treatment in *H. pylori* infections. Nowadays, use of probiotics is an emerging treatment option. They improve the eradication rates and side effects of the therapies used in *H. pylori* treatment by reducing *H. pylori* adhesion or colonization[49]. While a meta-analysis evaluating probiotics found increase in eradication rates with both Lactobacillus and Bifidobacterium, no significant improvement in side effects was seen[50]. A meta-analysis of nine studies on probiotic use as an adjuvant therapy found raise in eradication rates by 17%[51]. When Saccharomyces boulardii was added to *H. pylori* eradication regimens, decrease in side effects, especially in diarrhea, was observed as well as higher eradication rates[50,52]. Since the safety profile of probiotics is known, it is reasonable to suggest people under *H. pylori* therapy to eat yogurt.

Pre-treatment with n-acetlycysteine as a mucolytic agent is another approach to destroy the biofilm of *H. pylori* and overcome the antibiotic resistance[53]. In a randomized controlled trial done amongst patients with a history of at least four eradication failures, the eradication rates were found higher in the group who received n-acetylcysteine before a culture-guided regimen[54].

Simvastatin was used in a randomized controlled trial where the proposed mechanism of action was its anti-inflammatory effect other than its cholesterol lowering effect and the eradication rates were found to be increased but no improvement was noted in side effects[55].

The studies on vaccine against *H. pylori* still continue. A recent vaccine based on Cag A - Vac A - neutrophil-activating proteins was developed but although recognized by the host's cellular and humoral immune systems, limited immunogenicity was observed[56]. Altman *et al*[57] modulated *H. pylori* lipopolysaccharides chemically to enhance immunogenicity which enhanced antibody responses and a modest reduction in gastric *H. pylori* load when administered prophylactically. Still, there is no vaccine in use.

CONCLUSION

H. pylori is associated with multiple diseases and 100% eradication is still not possible. Even after a successful eradication, reinfection or recurrence can occur. The efficacy of standard triple therapy is decreasing whilst the bismuth quadruple and sequential regimen has been proven to achieve higher cure rates. Even though there are multiple guidelines (all therapies are summarized in Table 1) about the treatment regimens, increasing antibiotic resistance as well as different frequencies in resistance in different areas of the world suggests the optimal approach in the treatment of patients with *H. pylori* infections to be individualized therapy, which is a highly active and well-tolerated regimen. Use of probiotics, pre-treatment with n-acetylcysteine and statins may help as adjuvant therapies.

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EDITORIAL

Hepatocyte selection medium eliminating induced pluripotent stem cells among primary human hepatocytes

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Abstract

Hepatic insufficiency is a fatal liver disease with a significant decrease in functioning hepatocytes. If hepatocytes could be generated from human induced pluripotent stem (hiPS) cells and transplanted into patients with hepatic insufficiency, the disease may become curable. However, a major limitation to this therapeutic strategy is due to the tumorigenicity of hiPS cells and their ability to form cancer. Current methods for eliminating unwanted hiPS cells use genetic manipulation or reagents that are potentially hazardous for hepatocytes; therefore, revised methods are necessary and anticipated. Glucose and arginine are essential cell culture medium ingredients for the survival of most cells, including hiPS cells. However, hepatocytes can produce its own glucose and arginine through galactokinase and ornithine transcarbamylase, respectively. Therefore, it was hypothesized that unwanted hiPS cells could be eliminated in a medium without glucose and arginine, and supplemented with galactose and ornithine instead. This modified medium has been established as hepatocyte selection medium (HSM). So far, attempts to generate a pure colony of mature hepatocytes from hiPS cells have not been successful. After establishment of co-culture in HSM,

primary human hepatocytes survive while hiPS cells die within three days. Our latest results regarding a modification of HSM will be introduced in this manuscript.

Key words: Ornithine transcarbamylase; Galactokinase; Arginine; Galactose; Urea cycle

Core tip: Human induced pluripotent stem (hiPS) cells have the potential to differentiate into mature hepatocytes. If undifferentiated hiPS cells persist among transplanted hepatocytes, hiPS cells may potentially develop into cancer. Glucose and arginine are essential for the survival of most cells; however, mature hepatocytes survive in the media because they can produce glucose and arginine using galactokinase and ornithine transcarbamylase, respectively. Therefore, we created a hepatocyte selection medium (HSM) that lacks glucose and arginine but is supplemented with galactose and ornithine. After establishment of co-culture in HSM, human primary hepatocytes survive while hiPS cells die within three days.

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INTRODUCTION

Human induced pluripotent stem (hiPS) cells have pluripotency and potential to differentiate to various types of somatic cells. HiPS cells are established with four reprogramming factors[1].

Hepatic failure is fatal in that numbers of functioning hepatocytes significantly decreases below a safe level to support life. If hepatocytes are generated from hiPS cells and transplanted to patients with hepatic failure, they would be cured[2]. One major problem of transplantation is graft-verses-host disease. The graft-verses-host disease could be avoided, if hepatocytes are differentiated from hiPS cells from the patients.

One problem arises with the transplantation. HiPS cells may reside in the transplanted hepatocytes. If the residual hiPS cells are transplanted to the patients, they potentially form cancer. Therefore, methods for eliminating hiPS cells among differentiated hepatocytes used for transplantation are needed for development. The methods should be non-hazardous to hepatocytes and specific for hiPS cells. As such, a new medium to eliminate hiPS cells "hepatocyte selection medium" (HSM), has been developed.

HEPATOCYTE DIFFERENTIATION FROM HIPS CELLS

HiPS cells differentiation to hepatocytes has been investigated[3-8]. They are divided into two categories: growth factors and transcription factors.

Currently, the most common protocols are stepwise addition of growth factors to simulate the process of *in vivo* hepatocyte differentiation during liver development[3-6]. Transcription factors are sequentially expressed during hepatocyte differentiation[7]. HiPS cells are difficult to transfect with plasmid DNA and as such, adenovirus vectors are more efficiently used to introduce transcription factors to hiPS cells. These transcriptions factors then induce differentiation of hiPS cells into hepatoblast-like or hepatocyte-like cells[6,8]. However, there are limitations using this method and cells exhibit only certain similarities to primary hepatocytes and are therefore called "hepatocyte-like cells". An organoid of liver is formed after mixing human mesenchymal stem cells, human umbilical vascular endothelial cells, and hepatocyte-like cells differentiated from hiPS cells[9]. One major problem is that most attempts to obtain mature hepatocytes from hiPS cells have not been successful.

Another limitation, as described in further detail below, is the possibility of tumor formation and cancer development due to the presence of undifferentiated hiPS cells among transplanted hepatocyte-like cells.

TUMORIGENICITY OF HIPS CELLS

HiPS cells proliferate rapidly and have active telomerase activity, which are closely associated with tumorigenicity[1,10]. Tumorigenicity is therefore a major concern with the transplantation of somatic cells differentiated from hiPS or other stem cells into patients[11]. In fact, teratoma is formed in the liver transplanted with mouse hepatocytes differentiated from embryonic stem (ES) cells[12]. This phenomenon strongly supports the tumorigenicity of the residual undifferentiated mouse ES cells among the hepatocytes. At an early stage, it was speculated that teratoma was caused by viral vectors integrated to the host genome[13]. To reduce this risk, two methods have been attempted. First one is the Sendai virus and plasmid vectors because they do not integrate to the genome[14,15]. Second one is to omit c-Myc from the four reprogramming factors[16]. With all the above trials, pluripotent stem cells still form teratoma. It is, then, speculated that tumorigenicity is strongly associated with pluripotency[10,17]. Methods should be investigated to eliminate hiPS cells from the transplanted cells.

ELIMINATION OF HIPS CELLS

Until now, several methods are reported on the elimination

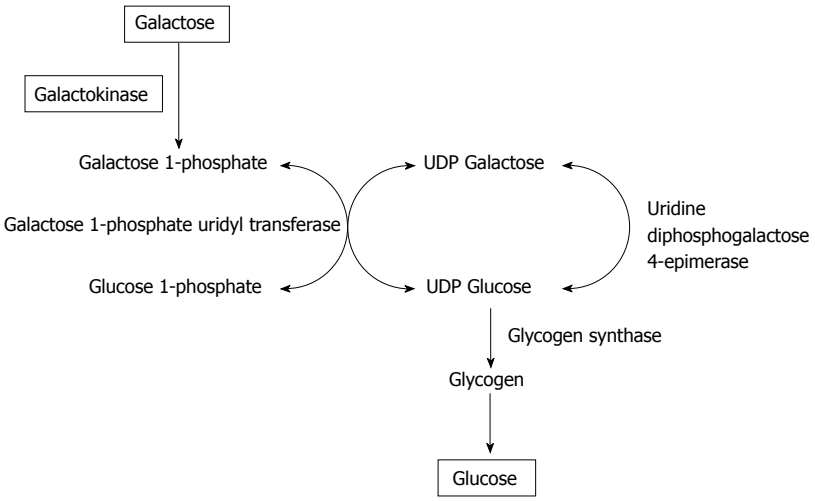

Figure 1 Gluconeogenesis from galactose. Galactose is converted to galactose 1-phosphate by galactokinase. The process of gluconeogenesis is shown and further described in the text. UDP: Uridine diphosphate.

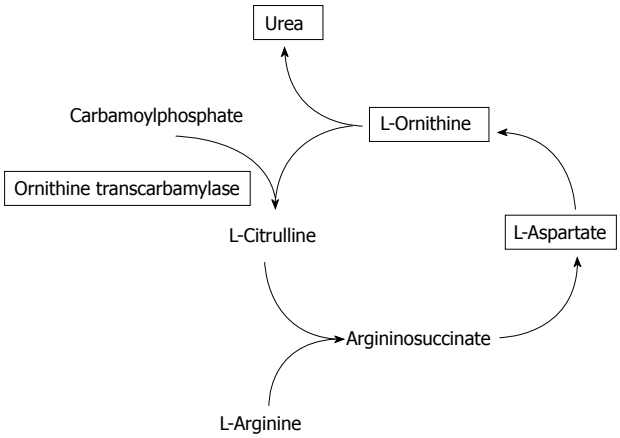

Figure 2 Urea cycle. L-ornithine is converted to L-citrulline by ornithine transcarbamylase. L-arginine is produced from L-ornithine by the urea cycle. Please see text for further detail.

of unwanted hiPS cells.

First attempt was introduction of thymidine kinase gene followed by addition of ganciclovir. Nanog is a homeodomain protein, and maintains pluripotency of ES cells[18]. Nanog promoter would drive thymidine kinase gene if the promoter is followed by the gene in ES cells. It would be expected that hiPS cell would be eliminated with ganciclovir. As expected, hiPS cells die after the addition of ganciclovir into the media[19]. Similarly, Lim *et al*[20] introduced the thymidine kinase gene with lentiviral vectors. However, the approach of selectively eliminating hiPS cells in this manner may be problematic because ganciclovir may be hazardous to hepatocytes.

Second attempt was small molecules. A library of small chemicals was screened to find molecules that had the potential of induction of apoptosis for mouse ES cells[21]. Benzethonium chloride and methylbenzethonium successfully induced apoptosis in hiPS.

N-oleoyl serinol (S18) is a ceramide analogue. S18 ablated pluripotent cells in EB differentiating toward neuronal lineage[22]. Unexpectedly, S18 promoted differentiation of embryoid bodies to neural lineage. S18 is unique in two aspects: elimination of hiPS cells and promotion of differentation toward neural lineage.

Altogether, the above methods require genetic modification or reagents that are hazardous to hiPS cells. However, genetic modifications and toxic reagents are not desirable for the transplantation of somatic cells differentiated from hiPS cells. Therefore, a method should be developed to eliminate hiPS cells using non-toxic materials.

GALACTOSE AS A SOURCE OF ENERGY

Glucose is indispensable for virtually all type of the cells to survive. Glucose is metabolized to pyruvate though glycolysis. Pyruvate produces energy through tricarboxylic acid cycle.

Figure 1 illustrates galactose metabolization to glucose. Galactose is catalyzed to galactose-1-phosphate by galackokinase (GALK). Galatcose-1-phosphate uridyl transferase changed galactose-1-phosphate to glucose-1-phosphate. Through this reaction, glycogen is synthesized. Glycogen enters glycolysis. Finally, glucose is produced from galactose. Glucose-1-phosphate is changed to glucose-6-phosphate. Glucose-6-phosphate is the first metabolite from glucose. Glycolysis follows glucose-6-phosphate. GALK is solely expressed in the liver and kidney[23,24]. In this sense, galactose is the same source of energy as glucose in hepatocytes due to GALK. Therefore, it is expected that hepatocytes survive in a medium without glucose or pyruvate, and added with galactose[25,26]. As expected, hepatocytes survive in a medium without glucose, and added with galactose[27].

GLUCOSE DEPRIVATION AND HIPS CELLS

Metabolomic profiling has shown that glycolysis is

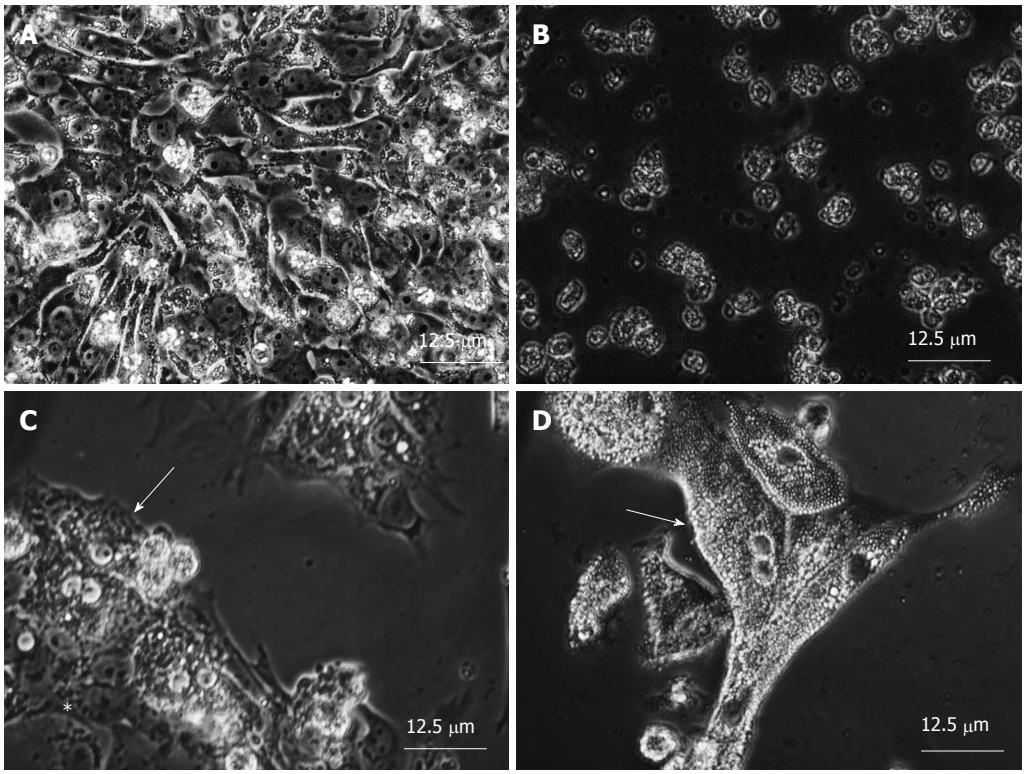

Figure 3 Co-culture of primary human hepatocytes and induced pluripotent stem cells. HiPS cells were cultured in Repro FF (Reprocell, Yokohama, Japan), a feeder free condition (A). The medium was changed to HSM, and cultured further for three days. All the iPS cells were eliminated (B). To address the possibility that hepatocytes survive and iPS cells were eliminated in HSM, co-culture of primary human hepatocytes (arrow) and hiPS cells (asterisk) were established (C). Currently, differentiation of hiPS cell to mature hepatocytes has not been successful. Primary human hepatocytes were used in place of hepatocytes from hiPS cells. HiPS cells were eliminated and primary human hepatocytes survived after three days culture in HSM (D). Original magnification: × 400. HiPS: Human induced pluripotent stem; HSM: Hepatocyte selection medium.

activated, glucose consumption is up-regulated, and lactate accumulation occurs in reprogrammed hiPS cells[28].

Glucose-depleted and lactate-enriched medium eliminated residual undifferentiated hiPS cells from induced differentiated cardiomyocytes[29]. Cardiomyocytes are able to obtain ATP from lactate while hiPS cells are not[30]. Using this non-genetic method, the authors have succeeded in selecting cardiomyocytes differentiated from hiPS cells with great purity and without forming tumors.

ARGININE AND THE UREA CYCLE

Arginine is important in production of nitric oxide polyamine[31]. Arginine is classified as a non-essential amino acid because the amino acid is produced by *de novo* synthesis. Cells require arginie to survive because its amount of production is not enough[32]. Cells are, therefore, hard to survive without argnine[33]. Culture media contain arginine for cells to survive and proliferate.

Arginine is produced through urea cycle. Arginine is normally produced in hepatocytes because urea cycle is expressed only in hepatocytes.

Urea cycle is important to detoxify ammonium ions produced from protein degradation[34]. Figure 2 maps metabolism of urea cycle. L-ornithine and carbamoylphosphate is metabolized to L-citrulline by ornithine transcarbamylase (OTC). OTC deficienty causes hyperammonaemic crises in neonates[35].

Niwa *et al*[27] cultured a rat hepatoma cell line, H4-IIE, in a medium without arginine. The cells were successfully cultured up to 30 passages. Interestingly, the surviving cells expressed all of the enzymes involved in the urea cycle. We could not find any literature addressing the involvement of the urea cycle in hiPS cells.

EXPRESSION LEVLES OF GALK AND OTC

In humans, two genes are involved in galactose metabolism: *GALK1* (NM_000154) and *GALK2* (NM_002044). *GALK1* is a major player of galactose metabolism, its deficiency causes cataracts in infants[36]. Whereas, *GALK2* was originally discovered to be involved in N-acetylgalactosamine metabolism[37]. However, in conditions with high concentrations of galactose, GALK2 demonstrates galactokinase activity.

HiPS cells express *GALK1*, *GALK2*, and *OTC* at significantly lower levels than fetal or adult liver[38]. It was therefore, expected that hiPS cells were eliminated in a medium without glucose or arginine.

HSM

Based on the discussions above, HSM was made to

eliminate mouse ES cells among cells differentiating toward hepatocyte lineage[39]. HSM was made from powder to omit glucose and arginine because the two ingredients are included in all the culture media commercially available. Formulation of HSM is based on Leibovits-15 medium that is suitable for the maintenance of function of cultured hepatocytes. HSM is not only deprived of glucose and arginine but also supplemted with galactose and ornithine. In addition, HSM is supplemented with proline for the synthesis of DNA in hepatocytes[40]. When HSM is applicable to human in the future, xeno-free condition is desirable. HSM, therefore, does not contain fetal bovine serum but knockout serum replacement (KSR) (Life Technologies, Grand Island, NY, United States) at 10%.

ELIMINATION OF UNWANTED HIPS CELLS AMONG PRIMARY HUMAN HEPATOCYTES

HSM was made as mentioned above. HiPS cells died within three days as expected in HSMs (Figure 3A and B)[38]. One concern arose: KSR. KSR might contain glucose, arginine, or both. To address this possibility, KSR was dialyzed, and compared with non-dialyzed one. HiPS cells died in HSM with or without dialysis. It was confirmed that hiPS cells die in HSM in three days. Theses encouraging results prompted us to culture hepatocytes in HSM. So far, differentiation has not been successful of hiPS cells to mature hepatocytes. Our HSM is not useful for generation of mature hepatocytes because the medium albated hiPS cells in three days culture. It, therefore, has not been successful to enrich mature hepatocytes differentiated from hiPS from undifferentiated hiPS cells. To overcome the limiting situation, primary human hepatocytes are subjected to co-culture experiments in place of hepatocytes successfully differentiated from hiPS cells. Figure 3C shows established co-culture of hiPS cells and primary human hepatocytes. Figure 3D clearly show that all the hiPS cells are eliminated and primary human hepatocytes survive in HSM[38].

POTENTIAL APPLICATION OF HSM

There are two ways of application of HSM. One is its initial aim to eliminate hPS cells among hepatocytes for transplantation. Another is application of HSM to hepatocyte differentiation.

One of the characteristics of HSM is that the medium does not have any toxic materials. Another characteristic of HSM is that it does not require genetic manipulation. HSM is, therefore, safe to eliminate unwanted hiPS cells. HSM is potentially necessary when patients with hepatic failure are transplanted with hepatocytes differentiated from hiPS cells to eliminated residual hiPS cells.

Kondo *et al*[41] report a medium that promotes hepatocyte differentiation from hiPS cells. The formulation of their medium is close to our HSM.

Recently, we have established a new medium based on HSM to initiate hepatocyte differentiation[42]. The medium is supplemented with an apoptosis inhibitor, oncostatin M, and small molecules. The report by Kondo *et al*[41] and our recent progress suggest that HSM may be a platform medium for differentiation of hiPS cells to hepatocytes.

CONCLUSION

HSM eliminates hiPS cells. HSM successfully isolates primary human hepatocytes from co-culture of hiPS cells and primary human hepatocytes. HSM may pave a way to a novel protocol to generate mature hepatocytes from hiPS cells.

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EDITORIAL

Metabolic bone disease in the preterm infant: Current state and future directions

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Abstract

Neonatal osteopenia is an important area of interest for neonatologists due to continuing increased survival of preterm infants. It can occur in high-risk infants such as preterm infants, infants on long-term diuretics or corticosteroids, and those with neuromuscular disorders. Complications such as rickets, pathological fractures, impaired respiratory function and poor growth in childhood can develop and may be the first clinical evidence of the condition. It is important for neonatologists managing such high-risk patients to regularly monitor biochemical markers for evidence of abnormal bone turnover and inadequate mineral intake in order to detect the early phases of impaired bone mineralization. Dual-energy X-ray absorptiometry has become an increasingly used research tool for assessing bone mineral density in children and neonates, but more studies are still needed before it can be used as a useful clinical tool. Prevention and early detection of osteopenia are key to the successful management of this condition and oral phosphate supplements should be started as soon as is feasible.

Key words: Premature; Osteopenia; Bone metabolism; Calcium; Alkaline phosphatase; Phosphorus; Nutrition

Core tip: Osteopenia of prematurity remains an important challenge in neonatal medicine due to continuing increased survival of preterm infants. The risk is higher with long-term diuretics or corticosteroids. It is important when managing such infants to regularly monitor biochemical markers for evidence of abnormal bone turnover and inadequate mineral intake. Dual-energy X-ray absorptiometry is increasingly used in research for assessing bone mass density in neonates. Prevention and early detection of osteopenia are key to the successful management of this condition and oral phosphate supplements should be started as soon as it is feasible.

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INTRODUCTION

Neonatal metabolic bone disease (MBD), osteopenia of prematurity (OOP), neonatal rickets or rickets of prematurity, are terms used to describe a reduction in bone mineral content (BMC) of the preterm infant. Although its exact prevalence is difficult to quantify because of the different methods used to screen infants at risk and also because of the difficulty in the interpretation of these results, it has been steadily increasing with the survival of more immature neonates as a result of advances in neonatal care. Born before a term pregnancy and thus deprived of a period of intrauterine supply of minerals, these infants already suffer at birth from suboptimal bone mineralization. The prevalence of MBD is inversely associated with birth weight and gestational age, with up to a third of infants weighing less than one kilogram at birth being osteopenic, more so if they are breastfed[1]. Other factors impeding normal bone mineralization include inadequate postnatal intake of vitamin D, calcium (Ca) and phosphorus (P), extended periods of total parenteral nutrition, lengthy duration of immobilization and as also a side effects of diuretics and corticosteroids prescribed to these infants[2,3]. Depending on the severity of the demineralization, osteopenia can remain clinically silent or develop as rickets, and, if severe, can even result in fractures[4].

As it is an important determinant of skeletal strength structure and density of the skeletal system throughout life, bone mineral density (BMD) in infants is an important topic for neonatologists, pediatricians and also endocrinologists. Guidelines for preventing, screening and treating MBD are not always consistent nor are they universally agreed upon, as still illustrated in a recently published review of this topic[5].

BURDEN OF OOP

Although the burden is not easy to quantify and available data remains conflicting, the known short-term complications are dominated by fractures of the long bones and ribs in the neonatal period. These respond well to therapy and there have no known residual long-term complications. The duration of hospital stay is unaffected by the diagnosis of OOP and preterm infants are routinely given mineral to prevent or treat neonatal rickets[6]. Growth alteration of the skull (dolichocephalic flattening) has been reported in association with poor BMC.

The weight, height, body mass index, lumbar BMC and BMD in 7-year-old children born prematurely and weighing less than 1500 g at birth are lower than those of the reference population[7]. Dual-energy X-ray absorptiometry (DEXA) assessment of areal BMD (aBMD; measured as grams per square centimeter) shows lower values at the level of the radial metaphysis, femoral neck and total hip in ex-preterm girls, but similar values at the radial and femoral diaphysis, with femoral neck aBMD remaining lower 12 mo later[8]. After adjusting for age, weight, height and jump power, prepubertal boys born at term have greater bone size and mass on DEXA scan at the age of at 5.7-8.3 years than those born before 34 wk of gestation[9]. It is still unknown if these changes in BMD in infancy and childhood increase the risk of developing early osteoporosis in adulthood.

PATHOPHYSIOLOGY AND RISK FACTORS

Antenatally

To develop normally, the skeleton of the growing fetus requires considerable active materno-fetal transfer of energy, protein, Ca and P. Serum Ca and *P* levels in the fetus are 20% more elevated than in the mother in the second trimester. Bone mineralization which occurs predominantly during the third trimester, will be inadequate if the fetal increased demands in Ca and P are not met. During pregnancy, augmented maternal intestinal absorption and increased skeletal mobilization increase maternal Ca supply to the fetus. The reduction in the Ca supply by the placenta results in a postnatal increase of parathyroid hormone (PTH) level that continues 48 h after birth when the peak serum Ca and the stabilization of serum Ca and *P* levels are attained[10,11].

Vitamin D also affects BMC and maternal hypovitaminosis D negatively affects the development of the fetal skeleton[12]. It is transferred across the placenta predominantly as 25-hydroxyvitamin D before conversion in the fetal kidney to the active form 1-25-dihydroxy-vitamin D.

Chronic damage to the placenta, with the resulting altered phosphate transport, also contributes to poor bone mineralization and explains the high postnatal incidence of rickets in neonates born with intrauterine growth retardation[13]. Such placental pathologies include pre-eclampsia[14] and also chorioamnionitis and placental infections[15].

Mechanical force patterns, such as fetal movements, including kicking against the wall of the uterus, also stimulate the growth of cortical bone[16]. As a result, preterm infants have a decrease in cortical bone growth leading to a reduction in bone strength. This, added to the reduction in transplacental accretion of Ca and P in the fetus, increases the risk of osteopenia in premature infants.

Postnatally

In infants who are exclusively breast fed, OOP is not

Table 1 Suggested guidelines for the prevention, monitoring and management of neonatal metabolic bone disease

Infants at risk	Prevention	Monitoring	Management
Born with birth weight below 1500 g Born before 28 wk of gestation	Early enteral nutritional intervention Maintain a sufficient supply of Ca and P. Start oral P supplements as soon as its feasible. The P absorption rate is very good in the presence of Ca, with absorption rates exceeding 90% with both human and formula milk. The Ca absorption rate increases from 35 to 60 mg/kg per day when both Ca and P are supplemented and to 90 mg/kg per day when the appropriate dietary Ca/P ratio is attained. High Ca and P retention rates are attained with high-mineral preterm milk formulae or with fortified human milk	Biochemical Monitor weekly serum "bone profile" (Ca, P and ALP): maintain serum Ca concentration between 2.05-2.75 mmol/L and serum P between 1.87-2.91 mmol/L If serum *P* < 1.8 mmol/L and ALP > 500 IU/L, renal TRP should be measured and, if it exceeds 95%, P supplementation should be started If serum P levels fail to increase and if serum ALP levels keep on rising, consider ergocalciferol or alphacalcidol therapy	If the biomarkers of MBD do not normalize, consider either vitamin D supplementation with up to 600 IU/d (although not well supported by evidence) or initiate instead ergocalciferol or alphacalcidol therapy in which case regular monitoring of urinary calcium/creatinine ratio is necessary to detect hypercalciuria
Having received total parenteral nutrition for more than four weeks	Vitamin D supplementation	DEXA	
On long-term diuretics or corticosteroid therapy	Ensure a minimum daily supplement of 400 IU vitamin D. Doses above 400 IU/d do not improve Ca and P absorption	Being increasingly used for assessing BMD in neonates, but not recommended as yet as a clinical tool	
Suffering from neuromuscular disorders	Parenteral nutrition Preparations providing 1.45 to 1.9 mmol/kg per day of Ca and 1.23 to 1.74 mmol/kg per day of P result in Ca and P retention rates of 88%-94% and 83%-97% respectively. The optimal Ca/P ratio in the intravenous solution fluid is between 1.3:1 and 1.7:1.54 If needed, parenteral P delivery can also be enhanced by using special preparations of organic P Exercises Daily exercises such as gentle compression and movements of the limbs Regular review of medications in use Discontinuation of diuretics and steroids when appropriate	Monitor for metabolic acidosis and hypercalciuria which may result from an increase in parenteral mineral delivery during parenteral nutrition	

ALP: Alkaline phosphatase; BMD: Bone mass density; Ca: Calcium; IU: International units; P: Phosphorus; TRP: Tubular reabsorption of phosphate. DEXA: Dual-energy X-ray absorptiometry.

correlated with the degree of the prematurity[17]. Very low birth weight infants (VLBW) whose full enteral feedings have been delayed delayed and who are on long term parenteral nutrition are at increased risk of OOP. Poor bone mineralization is also associated with common neonatal conditions. These include sepsis, broncho-pulmonary dysplasia, cerebral pathology, neuromuscular conditions leading to prolonged immobilisation, acidosis, necrotizing enterocolitis and also cholestasis. Frequently used medications such as diuretics, corticosteroids and methylxanthines also increase the risk of inadequate bone mineralization. Factors associated with increased BMD included higher birth weight, short duration of parenteral nutrition, absence of intraventricular hemorrhage, exclusive feeding of fortified breast milk, and older age at discharge[18].

Candidate genes associated with adult osteoporosis have recently been evaluated in VLBW infants where MBD was found to be associated with a lower number of thymidine-adenine (TA) repeats polymorphism of the estrogen receptor gene, compared to a higher number in those without MBD[19].

SCREENING AND MONITORING

As MBD is usually asymptomatic in most infants, its diagnosis depends essentially on screening. This is based on a set of criteria defined by the presence of clinical manifestations, radiologic findings, biochemical markers and BMC measurements. The recognized clinical-radiological associations include, bone demineralization, periosteal reactions and, in severe cases of osteopenia, rickets and pathological fractures may be present[20]. Infants at high risk of osteopenia, including VLBW infants or neonates on long term diuretic therapy should be regularly monitored for that condition as serious complications can be avoided by early diagnosis with appropriate management. Measuring BMC and BMD relies on a few surrogate markers (Table 1).

Serum biomarkers

As a normal serum Ca level can still be maintained to the detriment of Ca loss from the bone, it should not be used to screen infants at risk. Furthermore, serum Ca may also be affected by unrelated conditions such

as hypophosphataemia[16,21]. Serum P concentration is correlated with BMD, is highly specific but is not sensitive enough to identify infants with ostepenia. While serum P concentration adequately reflects *P* levels in the bone, serum Ca concentration remains maintained at the cost of Ca content in the skeleton.

Serum alkaline phosphatase (ALP) is a marker of bony turnover. Elevated levels indicate increased bone cellular activity and when exceeding 700 to 750 IU/L, they are associated with osteopenia, which is still asymptomatic at that stage[22,23]. The diagnosis of MBD in the preterm infant is usually suggested by the presence of low serum P levels in association with elevated serum ALP levels[1]. The association of serum ALP levels exceeding 900 UI/L with a serum *P* level less than 1.8 mmol/L is 100% sensitive and 70% specific to diagnose OOP[24]. A serum ALP level exceeding five times the upper limit of the normal range in adults is also associated with an increased risk of rickets[25]. The diagnosis of OOP cannot be made however with certainty by elevated serum ALP concentrations, because DEXA scan measurements of BMC did not find an association between ALP levels and OOP in some studies[26] and also because healthy preterm and osteopenic infants have higher serum ALP concentrations than full term infants. Associating multiple measurements of serum ALP with a wrist radiograph, with or without that of the knee, has been suggested for the identification of rickets in VLBW infants if the levels exceed 800 IU/L[27]. Because it is located on osteoblast surfaces, bone-specific ALP is a more specific biomarker of bone turnover, useful to confirm OOP, when high levels of total serum ALP are found[28,29]. Despite its limitations and, despite the absence of a clear cut-off diagnostic level, serum ALP measurement is frequently used to screen high risk infants for MBD. It is a readily available measurement in most laboratories and serial serum levels provide a trend very useful for follow up. Using it in conjunction with serum P levels as a screening tool significantly increases the sensitivity of identifying infants at risk of MBD.

Serum osteocalcin (OC), a non-collagenous protein of the bony matrix, is also a biomarker of osteoblastic activity. It is synthesized by osteoblasts and is partly regulated by 1,25-dihydroxyvitamin D levels. Its serum concentrations are elevated whenever bone turnover is increased, making it a possible useful tool to diagnose OOP[1]. However, despite its specificity, there is no correlation between serum OC levels and BMC in the first four months of age[30].

Urinary biomarkers

Urinary Ca and P excretion have also been used as biomarkers of postnatal skeletal mineralization. Urinary excretion of Ca exceeding 1.2 mmol/L and P exceeding 0.4 mmol/L are strongly associated with high bone mineralization. Infants born between 23 and 25 wk of gestation have a significantly lower renal P excretion threshold than other preterm neonates, resulting in elevated urinary P excretion even when serum *P* levels are low[31]. As, unlike Ca, P is not bound in the plasma, it has been suggested that a better biomarker for OOP is the percentage of renal tubular reabsorption of phosphate (TRP), with TRP > 95% indicating inadequate supplementation, bearing in mind that renal tubular leak of P can also be associated with inadequate Ca intake and increased serum PTH concentration[32]. Similarly urinary Ca or P to creatinine ratios may also be useful as biomarkers for OOP; normal reference ranges in preterm infants have already been established for these ratios[33,34]. However these urinary ratio results need to be carefully interpreted as they are highly dependent on the dietary intake (resulting in uncertainty if the standard reference range) and are also affected by the administration of drug such as furosemide or theophylline[35].

Radiological markers

Old fractures and cortical thinning may be seen on plain radiographs, reflecting poor bone mineralization, but are usually very late signs because they are not usually apparent unless the BMC decreases to 40%[36].

DEXA is currently the most widely used modality to assess BMD. It correlates well with fracture risk and, in both term and preterm infants, it can be used to estimate BMC[37]. Measuring BMD prior to adulthood however is hindered by the "areal" nature of the derived measurement. In addition, the establishment of robust, reliable neonatal, ethnic and gender specific normograms is urgently needed. Barriers to the routine use of DEXA as a screening tool for OOP include its high cost, its limited availability, the dimensions of the equipment used, the lengthy time required for imaging, as well as its sensitivity to movement artifact.

Quantitative Ultra sound (QUS), with already established reference values for both preterm and term infants, is a new inexpensive and portable modality of investigating OOP[38-40]. This simple, non-invasive and inexpensive bedside method measures the broadband ultrasound speed attenuation, and is usually performed on the tibia. Although the measurements it provides correlate well with bone density and structure, the association is a poor with the thickness of the bony cortex[41]. QUS is significantly lower in preterm infants than term infants and a significant correlation of QUS exists with serum ALP, supplementation with Ca, P and vitamin D as well as risk factors for reduced BMD[42]. The combination of longitudinal QUS measurements with serum ALP and *P* levels are helpful to identify infants at increased risk of OOP[43].

Although ultrasound reference values are available for term and preterm infants, there is limited information showing its usefulness.

PREVENTION AND TREATMENT

These are summarized in Table 1. The prevalence and also the severity of OOP can be reduced by early nutritional intervention. Maintaining a sufficient supply of

Ca and P for the growth of VLBW infants' skeleton is challenging because of their relatively high physiological requirements. In addition, although preterm infants are capable of absorbing up to 70% of Ca from human milk, the P content affects the Ca retention rate. Supplementing milk with both Ca and P is more effective: while the Ca absorption rate is 35 mg/kg per day in the presence of P supplementation alone, it increases to 60 mg/kg per day when both Ca and P are supplemented. Ca absorption is also affected by the dietary Ca/P ratio with the retention rate reaching up to 90 mg/kg per day when the appropriate ratio is attained. The neonatal intestinal absorption of P is very good in the presence of Ca, with absorption rates exceeding 90% with both human and formula milk[44]. Ca and P retention rates similar to those observed in utero are attained with high-mineral preterm milk formulae or with fortified human milk[45].

It is imperative to monitor closely serum Ca, P and ALP in such high-risk infants. To prevent OOP, serum Ca concentration should be maintained between 2.05-2.75 mmol/L and serum P between 1.87-2.91 mmol/L. Although VLBW infants are routinely given vitamin D supplementation to increase intestinal absorption of Ca and P, doses above 400 IU/d do not improve their absorption[46].

Parenteral nutrition preparations providing 1.45 to 1.9 mmol/kg per day of Ca and 1.23 to 1.74 mmol/kg per day of P result in Ca and P retention rates of 88%-94% and 83%-97% respectively, equivalent to 60% to 70% of the expected in utero Ca and P accretion rates[47,48]. Ca and P delivery by parenteral nutrition are affected not only by their respective concentrations in the intravenous solution, but also by the ratio of their concentrations. The optimal Ca/P ratio in the intravenous solution fluid is between 1.3:1 and 1.7:1.54[49-51]. The supply of these minerals to infants is limited by the poor solubility of both Ca and P in parenteral nutrition solution, resulting in an increase in the risk of OOP when enteral feeding is not possible for an extended period. Further research is required to improve Ca and P delivery with parenteral nutrition. Vigilance is required during parenteral nutrition as the increase in parenteral mineral delivery may result in metabolic acidosis and hypercalciuria[52]. If needed, parenteral P delivery can also be enhanced by using special preparations of organic P.

Because of the crucial role of mechanical forces on the development of the skeleton, daily exercises such as gentle compression and movements of the limbs are recommended in infants at risk of OOP if greater increase in body weight, forearm bone length, bone area and BMC are to be achieved[53-55].

CURRENT RECOMMENDATIONS

Guidelines for screening and treating infants at risk of OOP have been developed[56]. As summarized in Table 1, it is recommended to monitor all infants for MBD if their birth weight is below 1500 g, or if born before 28 wk of gestation, or if they have received total parenteral nutrition for more than four weeks or in case of diuretic or corticosteroid therapy. Monitoring consists of weekly serum "bone profile" (Ca, P and ALP). If serum P < 1.8 mmol/L and ALP > 500 IU/L, renal TRP should be measured and, if it exceeds 95%, P supplementation should be started. If serum P levels fail to increase and if serum ALP levels keep on rising, ergocalciferol or alphacalcidol therapy should be then considered. The American Academy of Pediatrics recommends that all breast-fed, partially breast-fed and non-breast-fed infants consuming less than 1000 mL of vitamin D fortified milk daily should be supplemented daily with a minimum of 400 IU vitamin D[57]. If the biomarkers of MBD do not normalize, vitamin D supplementation with up to 600 IU/d has been suggested, but without much supporting evidence. In addition, daily passive exercises should be encouraged and the medications in use should be regularly reviewed with discontinuation of diuretics and steroids when appropriate.

CONCLUSION

Preterm infants, those on long-term diuretics or corticosteroids, and those with neuromuscular disorders are at high risk of developing osteopenia. Complications such as rickets and pathological fractures may be the first manifestation of the condition. To detect the early asymptomatic phases of impaired bone mineralization and allow early intervention, all neonates at high risk of MBD appropriate biochemical markers of insufficient intake minerals and of abnormal bone turnover should be regularly monitored. DEXA is being increasingly used for assessing BMD in neonates, but more studies are still needed before it can be used as a useful clinical tool. Prevention and early diagnosis of MBD are key to the successful management of this condition and oral P supplements should be started as soon as is feasible.

Prospective studies of cohorts of preterm infants with OOP are needed with close long-term follow up for later outcomes. More research into urinary Ca and P to creatinine ratios is needed before they can reliably replace direct measurement of BMC. Similarly DEXA needs to be studied further to better define the "areal" nature of the measurement derived for BMD estimation in the newborn and also to establish reliable neonatal, ethnic and sex specific normograms. The possible role of QUS in routine screening for OOP needs also to be studied. As the poor solubility of Ca and P in parenteral nutrition solution hampers the adequacy of their supply to the growing newborn, further research in this area is required to increase their delivery.

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EDITORIAL

Methodological challenges to control for immortal time bias in addressing drug effects in type 2 diabetes

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Abstract

There are multiple biases in using observational studies to examine treatment effects such as those from prevalent drug users, immortal time and drug indications. We used renin angiotensin system (RAS) inhibitors and statins as reference drugs with proven efficacies in randomized clinical trials (RCTs) and examined their effectiveness in the prospective Hong Kong Diabetes Registry using adjustment methods proposed in the literature. Using time-dependent exposures to drug treatments yielded greatly inflated hazard ratios (HR) regarding the treatment effects of these drugs for cardiovascular disease (CVD) in type 2 diabetes. These errors were probably due to changing indications to use these drugs during follow up periods, especially at the time of drug commencement making time-dependent analysis extremely problematic. Using time-fixed analysis with exclusion of immortal time and adjustment for confounders at baseline and/or during follow-up periods, the HR of RAS inhibitors for CVD was comparable to that in RCT. The result supported the use of the Registry for performing pharmacoepidemiological analysis which revealed an attenuated low low-density lipoprotein cholesterol related cancer risk with RAS inhibitors. On the other hand, time-fixed analysis with including immortal time and adjustment for confounders at baseline and/or during follow-up periods, the HR of statins for CVD was similar to that in the RCT. Our results highlight the complexity and difficulty in removing these biases. We call for validations of the methods to cope with immortal time and drug use indications before applying them to particular research questions, so to avoid making erroneous conclusions.

Key words: Pharmacoepidemiological analysis; Immortal time bias; Drug effects; Prevalent drug user bias; Drug indication bias; Type 2 diabetes

Core tip: There are multiple biases in using observational studies to examine treatment effects. These biases include those due to prevalent drug users, immortal time and drug indications that must be taken into consideration. In this regard, we used drugs with proven effects in randomized controlled trials and applied those proposed methods by other groups to estimate their effects in a prospective cohort of patients with type 2 diabetes. Our results highlighted the importance of validating adjustment methods for immortal time and drug use indications before applying them to addressing research questions, so to avoid making erroneous conclusions.

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INTRODUCTION

In pharmacoepidemiological analysis, there are multiple biases in using observational studies to examine treatment effects. These biases may be due to prevalent drug users, immortal time and drug indications[1]. Given these biases, the question to ask is, do we have a way to judge whether the quality of the database of an observational study or the method of analysis is free from these three major biases?

The prevalent user bias is easy to discern and can be readily excluded during data analysis. In type 2 diabetes (T2D), biases due to drug indication depend upon whether the subphenotypes associated with the drug usage (indications) may have selected a patient subgroup inherently at high risk for a clinical outcome, *e.g.,* cardiovascular disease (CVD) or cancer. Immortal time bias is not difficult to detect because we can suspect such a bias as long as immortal time is reported, *i.e.,* non-drug exposure periods have been classified as exposure periods. Several methods have been proposed to control for immortal bias but it is uncertain whether these methods can adequately remove these biases[2-4]. Our recent work has shed light on these important issues[5,6].

INDICATIONS OF DRUG USE AND CONFOUNDERS

Hyperglycemia is the reason why a person is prescribed an antidiabetic regimen which can include various combinations of oral antidiabetic drugs (OAD), insulin and other injectables, such as glucagon like peptide 1[7]. Besides, many factors such as disease severity, predominant disease mechanisms (*e.g.,* insulin deficiency versus insulin resistance), prescribing habits, formulary restrictions, willingness to pay for or accept treatment, referral and volunteer biases can also affect the choices of drug combinations as first, second or third lines of treatments during the clinical course. Of note, some of these factors which can influence drug choices may not be captured in observational studies. Hence, randomized clinical trials (RCT) remain to be the gold standard by evenly distributing these unmeasured confounders in different experimental and control groups to reduce these biases.

Several studies including ours have reported an association of hyperglycemia with cancer in diabetes[8,9]. Our group also reported a linear association between glycated hemoglobin (HbA_{1c}) and all-site cancer in T2D with 1% increase in HbA_{1c} associated with 18% increase in the risk of cancer[10]. These observations were supported by a meta-analysis of RCT data where 0.5% reduction in HbA_{1c} was associated with a non-significant hazard ratio (HR) of 0.91 for cancer risk in T2D[11]. In a recent large randomized trial[12], treatment with saxagliptin, a dipeptidyl peptidase 4, was associated with 0.3% reduction in HbA_{1c} accompanied by a 50% reduction in the risk of pancreatic cancer, albeit short of significance[12]. Although the underlying mechanism linking hyperglycemia and cancer remains to be elucidated, the overarching premise is that users of OADs and insulin are high risk subjects for cancer. Unless these drug indications are captured and removed, these drug users are likely to be found to increase cancer risk, which might be erroneously attributed to drug effects.

In epidemiological analysis, propensity score is often used to control for indications of drug use[13]. The robustness of these scores in removing selection bias is indicated by the area under receiver's operating characteristics curve (AUC) where values $\geqslant 0.90$, $\geqslant 0.80$ to < 0.90, and $\geqslant 0.70$ to < 0.80 indicate excellent, good and fair performance, respectively[1]. Apart from including propensity score, multivariable analysis with inclusion of subphenotypes associated with a clinical event, *e.g.,* cancer, can also attenuate bias due to drug indications[1]. In prospective cohort analysis of the Hong Kong Diabetes Registry, we had identified a group of subphenotypes for cancer risk in T2D[14], in addition to age and hyperglycemia. These included (1) body mass index $\geqslant 27.6$ kg/m^2 and $<$ 24 kg/m^2[15]; (2) low-density lipoprotein cholesterol (LDL-C) $\geqslant 3.8$ mmol/L[16]; (3) co-presence of LDL-C < 2.8 mmol/L and albuminuria[17] which was further enhanced in the presence of increased high density lipoprotein cholesterol (HDL-C) $\geqslant 1.0$ mmol/L[18]; (4) co-presence of LDL-C < 2.8 mmol/L and triglyceride < 1.7 mmol/L[18]; and (5) HDL-C < 1.0 mmol/L[19].

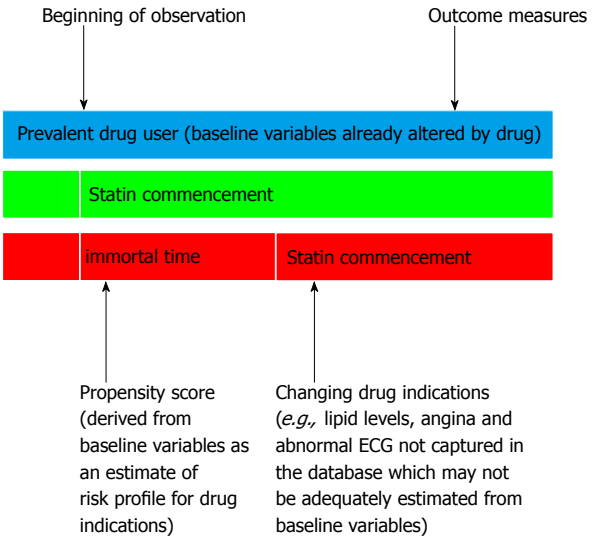

Figure 1 Schematic diagram to explain the multiple biases in epidemiological analysis of cardiovascular disease with use of statins in observational cohorts. While baseline variables may be used to derive a propensity score to adjust for drug indications, this will not apply to prevalent drug users whose risk profile has already been influenced by drug use (blue panel). For patients who were started on the drug after a period of immortal time (non-drug exposure), insufficient data capturing during the observation period may not allow full adjustment for confounders resulting in an inflated hazard ratio of cardiovascular disease, while inclusion of immortal time in a time-dependent Cox model may lead to a reduced hazard ratio by including a period of non-drug exposure during estimation of event rates (red panel). In patients with detailed documentation of risk factors followed by drug commencement as in a clinical trial setting, time fixed analysis may yield hazards unbiased by immortal time (green panel). ECG: Electrocardiogram.

IMMORTAL TIME AND IMMORTAL TIME BIAS

Immortal time refers to a period in cohort studies when non-exposure to a drug treatment from the baseline to the time of initiation of the drug treatment in the "drug exposure group" is misclassified as exposure to the drug treatment[1]. This misclassification may lead to a deflated HR of the treatment for the endpoint due to addition of the non-drug exposure period into the drug exposure period. This can lead to a false conclusion that the drug reduces the risk of the event of interest. Several researchers recommended the use of time-varying or time-dependent drug exposure Cox proportional hazard regression to cope with immortal time bias[3,4]. The use of this method assumes that exposure to the drug treatment or the drug commencement is at random[20]. However, this is rarely the case in real world practice since patients usually start on a drug treatment for a new or changed indication which may not be systematically captured in analysis of cohort study data. If these confounders are not available, the use of a time-dependent model may lead to an increased HR of the treatment for the endpoint (Figure 1).

In order to test the validity of these methods[3,4], we used a referent drug with proven benefits [*e.g.,* statin or renin angiotensin system (RAS) inhibitors] and applied the methods to the Hong Kong Diabetes Registry[5,6] to find out if the estimated effect size fell within the bounds of that reported in RCTs with regard to their associations with CVD. We tested various combinations of exclusion/inclusion of immortal time and adjustment for drug indications at baseline or at the end of immortal time when drug was commenced Consistently, time-dependent drug-exposure Cox models severely inflated the HR of these two drugs for CVD risk[5,6], despite their proven cardioprotective effects in RCTs. In the statin-CVD validation[6], compared to a HR of 0.63 (95%CI: 0.48 to 0.83) in a RCT[21], exclusion of immortal time and adjustment for estimated covariables at the end of the immortal time when statins were commenced, resulted in a 52.3% inflation in the HR of statins for CVD (0.96, 0.72 to 1.27), which was above the higher bound of the 95%CI. On the other hand, inclusion of immortal time, *i.e.*, ignoring immortal time bias, and adjustment for covariables at baseline generated the least inflated HR of 0.64 (0.48 to 0.84) which was within the HR estimates in clinical trial and inflated by 1.59% compared to the absolute HR of 0.63.

In the RAS inhibitors-CVD validation[5], exclusion of immortal time and adjustment for covariables when RAS inhibitors was commenced resulted in a HR of 0.89 (0.68 to 1.17) which was within the estimates of 0.92 (0.84-1.0) reported in RCTs with 3% deflated risk compared to the absolute hazard. By contrast, inclusion of immortal time and adjustment for covariables at baseline yielded a HR of 0.66 (0.51 to 0.86) which was outside the estimates with a 28.3% deflation rate (or inflation rate: -28.3%) compared to the absolute HR.

In most observational or administrative databases, the events preceding the commencement of drugs like statins (*e.g.,* high LDL-C, angina, abnormal imaging) were often not available in the dataset. In a time-dependent model which includes immortal time, inadequate adjustment for indications at the time of drug commencement can lead to overinflated hazards. In this situation, a non-time-dependent analysis but ignoring immortal time and adjusting for propensity score using covariables at baseline might yield the least bias. It is also possible that inflated hazards due to inadequate removal of drug indications and reduced hazards by including the immortal time might have cancelled out one another, giving a HR close to that in a RCT. In the case of drugs with more general indications such as RAS inhibitors, a time-fixed Cox model with exclusion of immortal time and adjustment of covariables at the end of the immortal time, estimated from the baseline variables, might remove most, if not all, of these biases.

Our results highlight the challenges in removing bias from drug indications and immortal time simultaneously if these biases have not been systematically captured. In this new era of big data, clearly, more research is needed to develop methods for removing immortal time bias. This is especially relevant to drugs such as statins and insulin, often prescribed for clinical conditions (*e.g.,* angina, poor glycemic control), the information of which

may not be documented in the database. Pending better methodologies, we recommend the use of non-time-dependent model with exclusion of immortal time and adjustment for propensity score or subphenotypes associated with the event of interest to reduce potential biases.

On the other hand, by selecting high quality datasets with documentation of drug usage and prognostic variables, pharmacoepidemiological analysis may uncover novel hypothesis for further testing. In an analysis of the diabetes-cancer link, in light of the phenotypic heterogeneity, we first used multivariable analysis to identify risk factors or subphenotypes associated with cancer. By adjusting for a low LDL-C related cancer-subphenotype at drug commencement, we discovered a novel drug-subphenotype interaction where RAS inhibitors specifically attenuated low LDL-C related cancer risk in T2D[22]. These pharmacoepidemiological findings, coupled with pathophysiological knowledge and evidence from mechanistic investigations, have provided the basis for a hypothesis where the complex cross-talk between the RAS and the insulin-like growth factor 1-cholesterol pathway might explain the diabetes-cancer link, for further testing[14].

CONCLUSION

In pharmacoepidemiological analysis, there are methodological challenges in removing biases from immortal time and drug indications simultaneously. Hence, risk associations between drug use and clinical events based on observational studies must be interpreted with great caution. To avoid misinterpretation, researchers should take these biases into consideration at the stage of study design, *e.g.,* by documenting indications or variables at the time when drugs are introduced or changed. Our validation studies indicated that exclusion of immortal time in an analysis testing effects of RAS inhibitors while inclusion of immortal time in an analysis testing effects of statins on CVD, respectively yielded effect sizes in T2D close to those obtained in RCTs. Our findings call for further research in developing methodology to simultaneously remove immortal time bias and drug use indication bias. Meanwhile, in the absence of methods which can address effects of different drugs in multiple databases, it will be prudent to use reference drugs and test the quality of databases and adjustment methods for immortal time and drug indications before testing of other drug associations with clinical outcomes to avoid erroneous conclusions.

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REVIEW

Endoscopic management of adenomatous ampullary lesions

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Abstract

Lesions of the ampulla of Vater represent an uncommon group of gastrointestinal malignancies. The majority of lesions of the ampulla of Vater are either adenomas or adenocarcinomas. Ampullary lesions are often incidental findings. Accurate preoperative diagnosis and staging of ampullary tumors is imperative for predicting prognosis and determining the most appropriate therapeutic approach. Endoscopic ampullectomy is a safe and efficacious therapeutic procedure that can obviate the need for potentially major surgical intervention. This review will provide the framework for the diagnosis and management of ampullary lesions from the perspective of the practicing gastroenterologist. Strategies for safe and successful endoscopic ampullectomy with a focus on accurate preoperative diagnosis and staging, resection technique, and management of complications are presented.

Key words: Papillary tumors; Endoscopic ampullectomy; Endoscopic ultrasound; Ampullary adenoma; Pancreatitis

Core tip: Adenomatous ampullary lesions are rare. Endoscopic retrograde cholangiopancreatography and endoscopic ultrasound (EUS) have changed the management of patients with these lesions. Endoscopic ampullectomy is a technique that has revolutionized the treatment of these lesions avoiding potential complications of surgery. We herein discuss the epidemiology, the role of EUS in the local staging and the role of endoscopy in the treatment of the adenomatous ampullary neoplasms.

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INTRODUCTION

The anatomy of the ampulla of Vater is complex. Ampullary adenomas (AA) are an uncommon group of gastrointestinal malignances. With the advances in esophagogastroduodenoscopy and ultrasonography, detection of ampullary neoplasms has increased. Most periampullary lesions are malignant tumors appearing from the ampulla, duodenum, or pancreas. Benign neoplasms entail in this region only < 10% of neoplasms[1-3]. Advances in endoscopic retrograde cholangiopancreatography (ERCP) and endoscopic ultrasound (EUS) have changed the clinical management of these patients. Endoscopic ampullectomy may be considered in patients with smaller lesions that do not contain invasive carcinoma, and in patients who are poor surgical candidates[4-6]. Many series have reported low morbidity and mortality with endoscopic therapy[4,7-19]. Detailed preoperative assessment and staging is needed in other to decide on the best therapeutic option. We review the epidemiology, the role of EUS, ERCP and endoscopy in the approach of ampullary neoplasms.

EPIDEMIOLOGY

Ampullary neoplasms comprise several lesions: adenoma, adenocarcinoma, adenoendocrine carcinoma, small cell carcinoma, adenosquamous carcinoma, and undifferentiated carcinoma[20]. Adenomas or adenocarcinomas representing > 95% of these lesions[21,22]. AA are benign lesions but, can potencially develop into ampullary carcinomas in a comparable progression to that of colorectal cancer[2,3,23-29]. AA can be sporadic or in the context of a familial polyposis syndromes [*e.g.*, familial adenomatous polyposis (FAP)]. FAP is a risk factor; 80% of affected patients develop duodenal adenomas, which are often multiple[30]. In this polyposis syndrome, the lifetime incidence of peri-AA is 50%-100%. The prevalence of AA has increased in the last years with the extensive availability of endoscopy.

CLINICAL MANIFESTATIONS

Ampullary lesions are often found incidentally on cross-sectional imaging or by endoscopic examination. Presenting symptoms are usually non-specific, reflecting biliary or pancreatic obstruction. The most common presentation is with painless jaundice, which is present in 50%-75% of patients[21,31-34]. Cholangitis or acute pancreatitis are rare manifestations[35-38]. Nausea, vomiting, biliary colic, and weight loss may also occur[21,33].

ACCURATE PREOPERATIVE DIAGNOSIS AND STAGING

Accurate preoperative diagnosis and staging is critical to decide on the best treatment option and establish a prognosis.

Endoscopy

The best endoscopic examination of the papilla of Vater is performed with a side-viewing endoscope[20]. This endoscope allows an adequate assessment of the morphological features of the lesion. Thus the following features are suggestive of benign disease: (1) a regular margin; (2) absence of ulceration or spontaneous bleeding; and (3) a soft consistency[39]. Furthermore, the side-viewing endoscope enables an easy acquisition of tissue by biopsy, at the time of procedure. However, on this respect, we know that sensitivity with forceps biopsies for demonstrating the presence of adenoma is > 90%; this is lower for adenocarcinoma, and there is up to 30% of miss diagnosis[11,40-42]. Thus, a negative histological diagnosis of carcinoma on endoscopic biopsy of an ampullary adenoma does not exclude a possible focus of adenocarcinoma[42-47]. The accuracy of endoscopic biopsies can be enhanced when additional techniques are employed. Thus, taking biopsies several days after sphincterotomy[48], and taking at least six biopsies, minimizes the chance of false negative results[49] . Despite its gaps, endoscopic forceps biopsy is the mainstay of pre-excisional histological assessment in lesions of the ampulla. However, we ought to remember that resection of all AA might be the best approach for excluding the presence of carcinoma.

Endoscopic retrograde cholangiopancreatography

ERCP has a central role in the staging and management of obstructive jaundice in AA. Adenoma ingrowth into the pancreatic or biliary ducts does not always indicate malignancy, but may hinder endoscopic excision and considerably decreases the chance of complete endoscopic resection. ERCP at the time of endoscopic papillectomy permits: (1) evaluate the intraductal extension; (2) the placement of a pancreatic stent in order to reduce the risk of pancreatitis; and (3) deploy, if required, a biliary duct stent for the palliation of obstructive jaundice.

EUS

EUS, in conjunction with ERCP, allows to assess for infiltration of the periampullary wall layers and pancreatobiliary ducts but, it does not have to be universally incorpored into the diagnostic evaluation of an ampullary adenoma[45,50-57]. The use of EUS in the assessment of AA is undefined. There is no consensus on the requirement or not for EUS prior to consideration of treatment on all patients with AA. It has been suggested by some experts that EUS is not required if the lesion is less than

1 cm in diameter or there are no endoscopic signs to suggest malignancy[58]. Others claim that, if accessible, EUS testing ought to be taken into consideration prior to endoscopic or surgical resection[59]. EUS has been reported to be of help in recognizing non-invasive lesions amenable to local resection, but as yet there are no preoperative test which are as accurate as clinical judgment and intraoperative pathological diagnosis[45,60]. A recent retrospective review concluded that EUS is useful in predicting the depth of mucosal invasion in the preoperative evaluation of suspected peri-ampullary and duodenal adenomas (specificity: 88%; negative predictive value: 90%)[53]. However, EUS is an invasive technique, operator dependent, with different rates of over-diagnosis and under-diagnosis. In this context, peritumoral inflammatory changes can lead to over-staging and likewise focal pancreatic infiltration to under-staging[61,62]. A recent meta-analisis of 14 studies and a systematic review, concluded that the results obtained by EUS were comparable to the histological results with moderate strength of agreement in the following: preoperative staging of papillary neoplasm, predicting lymph node involvement and tumor invasion[63]. The modest EUS sensitivity (77%) and specificity (78%) in predicting T1 neoplasms makes it not optimal in choosing the right patients for endoscopic papillectomy. EUS sensitivity and specificity for detecting nodal invasion was 70% and 74%, respectively. We believe, as other authors that if the clinical suspicion for invasive carcinoma is low (*e.g.*, absence of jaundice, endoscopic features of noncancerous lesion), and the lesion appears amenable to endoscopic resection, then EUS may not impact the endoscopist's decision to stage the lesion *via* ampullectomy. Few studies have been reported comparing efficacy of EUS and intraductal ultrasound (IDUS) for ampullary neoplasms[54,60,64]. IDUS was superior to EUS in terms of tumor visualization and staging (staging accuracy: 78%-93%). Therefore, IDUS can be particularly appropriate in deciding which patients should undergo endoscopic ampullectomy. However, the availability of this technique is limited and therefore the number of patients undergoing IDUS is small.

Magnetic resonance imaging and computed tomography

Magnetic resonance imaging and computed tomography (CT) use is limited to staging of know ampullary cancers, for nodal staging and metastatic evaluation. CT is less precise than EUS for T staging of ampullary cancer[56,65].

ENDOSCOPIC AMPULLECTOMY

Patients diagnosed with an ampullary adenoma have three treatment options: pancreaticoduodenectomy (Whipple procedure), surgical local excision (surgical ampullectomy), or endoscopic ampullectomy. There are no clear guidelines about the surgical or endoscopic management of AA and, if they should undergo postprocedure surveillance[66]. Surgical excision is typically recommended for patients with larger lesions, lesions that contain carcinoma, lesions with lymph node involvement on preprocedure imaging, or for patients who do not have access to an experienced endoscopist in ampullectomy. Pancreaticoduodenectomy is more likely to achieve complete excision compared with local excision, but it is associated with higher operative morbidity and mortality rates (25%-65% and 0%-10%, respectively)[67,68]. Perioperative mortality rates were lowest (< 4%) in centers with a high procedure volume. Surgical ampullectomy has lower morbidity and mortality, but has the disadvantage of having more recurrence rate. Randomized trials comparing surgical ampullectomy with pancreaticoduodenectomy have not been performed. Endoscopic ampullectomy was first described in 1983 by Suzuki *et al*[59] and ten years later Binmoeller *et al*[4] described a considerable case series. More recently, many other series have reported low morbidity and mortality with endoscopic therapy[7-19]. However, the role of endoscopic ampullectomy is still debatable and it is largely performed only in reference hospitals with skill in therapeutic endoscopy. Endoscopic ampullectomy may be considered in smaller lesions (< 30 mm) that do not contain carcinoma and in patients with severe diseases. Lesions with endoscopic characteristics suggestive of posible malignancy (*e.g.,* nonlifting, firmness, ulceration, friability) should be offered surgical resection[6].

ENDOSCOPIC AMPULLECTOMY TECHNIQUE

General principles

Endoscopic ampullectomy is a therapeutic modality which must be undertaken by an endoscopist with enough training and expertise. The goal with AA is for total en-bloc removal of the neoplasm. Initially, the endoscopist must determine whether resection of the entire lesion in one piece ("*en bloc*") is feasible and locate the margins of the lesion. This method has several advantages: (1) it increases the likelihood of complete removal; (2) it provides clear margins for histopathologic evaluation; and (3) it reduces the procedure time. However, *en bloc* excision may not be technically feasible if the adenoma is of a large size, and/or there is a limited endoscopic accessibility. Piecemeal excision is usually reserved for these cases, frequently with adjuvant ablative therapy[69]. It has been postulated that this technique can reduce recurrence rates, bleeding and perforation. However, comparative trials are lacking[13] (Figure 1).

Submucosal lifting

The role of submucosal injection of saline, which may be combined with epinephrine or methylene blue before ampullectomy, is controversial[6,62,66]. Epinephrine

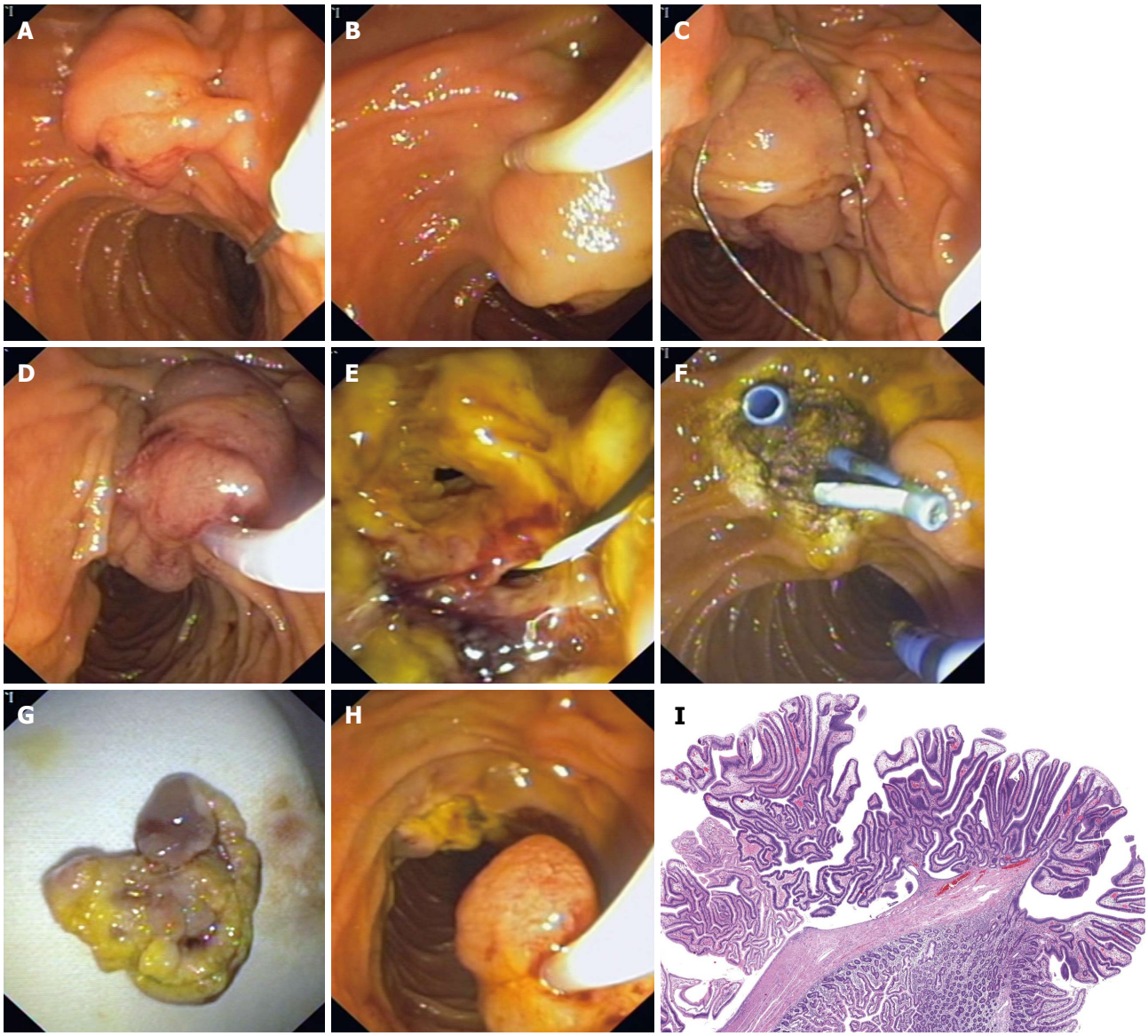

Figure 1 Technique of en-bloc ampullectomy. A: Lesion is identified; B: Submucosal (saline + epinephrine) injection; C: With the snare tip anchored above the papillary mound the entire papilla is snared; D: Check mobility and ensure the snare is firmly closed; E: En-bloc ampullary resection. Biliary and pancreatic (guidewire) orifice is identified; F: Biliary and pancreatic stents are placed. Adjuvant APC therapy is applied; G: Tissue retrieval with the snare; H: Ampullectomy specimen; I: Ampullary adenoma: tubulovillous architecture that shows neoplastic epithelial cells with pseudostratified and enlarged hyperchromatic nuclei. Adjacent there is normal duodenal mucosa. (HE: 20 ×). (Courtesy of Mercedes Hernando, MD). APC: Argon plasma coagulation.

and methylene blue may help minimize bleeding and enhance endoscopic visualization of the lesions margins, respectively[13]. Local saline injection may increase technical success and decrease complications similar to mucosectomy[13,70]. However, this technique is not recommended by other authors because submucosal saline injection may involve certain disadvantages: (1) the ampullary lesion may not lift due to tethering by the biliary and pancreatic ducts; (2) The dome effect created by submucosal injection may cause difficulty in the placement of the snare for effective *en bloc* resection[13,70-72]; and (3) increased risk of postresection pancreatitis has been reported. Currently, the evidence to support submucosal injection before ampullectomy is not significant. A possible indication may be adenomas with lateral extraampullary spread[72].

Endoscopic resection

There is no specific type of snare for endoscopic ampullectomy. For the majority of usual adenomas both hexagonal or oval snares of 3 cm are recommended. Standard braided polypectomy snares are typically used. The use of a thin wire snare is advised by some authors, limiting dispersion of the energy and risk of injury to the pancreatic orifice[72]. Occasionally, a peripheral circumferential incision to the adenoma with a needle knife device may make easier the snare capture[6]. To resect the lesion, the tip of the snare is placed on the top of adenoma; then, the snare is closed maximally and, after previously checking for papilla mobility, the lesion is

sectioned by continuous application of current.

Optimal current

There is no general recommendation regarding the optimal current and power output for endoscopic ampulectomy. Some investigators recommend pure-cutting current for this purpose[4,15,73] to preclude the edema originated by the coagulation mode, although, a pure cutting current has been related to bleeding. Others, using a blended electrosurgical current[4,6,9] or alternating cut/coagulation modes[6,62,74]. Power output oscillates between 30 W and 150 W[6,9,13,73,75]. Most experts, advocate a blended current[76]. We prefer to use Erbe electrosurgical generators (Endocut, effect 2)[77].

Retrieval of resected specimen

Retrieval of the specimen is essential for total evaluation and detection of small malignant foci. An anti-peristaltic agent administration (*e.g.*, glucagon or hyoscine butylbromide) to avoid intestinal migration is recommended. Retrieval should be performed immediately after excision since there is a tendency for the excised specimen to migrate distally into the jejunum. For this purpose, the snare that was used for the excision or a retrieval net is ideal. Endoscopic suction can also prevent the tissue migration. However, the specimen should not be aspirated through the accessory channel of the duodenoscope into a trap because this could lead to fragmentation of the specimen. Once retrieved, the specimen can be pinned to a polystyrene block to aid orientation and facilitate margin analysis.

Residual tissue ablation

After specimen retrieval, the duodenoscope is reintroduced to examine the resection site for: (1) active bleeding or bleeding stigmata; (2) residual tissue ablation. Usually, ablation therapy is used as adjunctive therapy to treat residual adenomatous tissue remaining after, *en bloc* or piecemeal, snare resection. With piecemeal excision, the tissue near the duct holes may be hard to excise completely. However, the benefits of this adjunctive therapy remain controversial. The overall success rate was comparable in patients with and without adjuvant thermal ablation (81% *vs* 78%, respectively)[9]. Ablation can be performed with monopolar or bipolar coagulation[49,70], and others devices[11,13,70,78]. We often use argon plasma coagulation (APC) (setting of 40 to 50 watts) to ablate residual tissue. We carry out a biliary sphincterotomy prior to fulguration, and we place a pancreatic stent before thermally coagulating around the pancreatic orifice.

Sphincterotomy and stent placement

The aim with a pancreatic or biliar sphincterotomy and stent placement is to enhance the technical success and decrease the complications of endoscopic ampullectomy[4,13,70,79-81]. However, a preresection sphincterotomy has some drawbacks. First, *en bloc* resection can be more difficult and will hinder total histologic evaluation of the resected specimen as result of thermal injury. Secondly, it may increase risks of bleeding, perforation and tumor seeding[82].

Usually, a meticulous inspection of the ampullectomy site allows identification of focal biliary and pancreatic orifices within the duodenal wall. Otherwise, secretin administration can produce juice flow to identify the pancreatic orifice. A 5 French pancreatic stent placement is advised to decrease the incidence and severity of pancreatitis[6,9,81,83,84]. Therefore, pancreatic duct stenting after endoscopic ampullectomy appears recomendable[74]. If ERCP or prior MRCP have demonstrated a pancreas divisum, pancreatic duct stenting is usually not necessary. Acute cholangitis after papillectomy is uncommon[76], and prophylactic biliary stent placement is generally unnecessary. However, we often perform either a biliary sphincterotomy or a prophylactic biliary stent is placed to minimize this probability. Biliary stenting may ensure the correct bile drainage if major bleeding occurs. The pancreatic and biliary stents are generally removed two or three weeks later, at which time any suspicious-appearing residual polypoid tissue can be removed to ensure complete excision.

COMPLICATIONS OF AMPULLECTOMY

Complications after endoscopic ampullectomy include bleeding (0%-25%), pancreatitis (0%-25%), perforation (0%-4%), papillary stenosis (0%-8%) and cholangitis (0%-2%)[4,6,9,11,13,62,85-87]. Pancreatitis, perforation and delayed bleeding are the most severe complications[62]. The overall complication rate is around 15%[4,11,49,70,80]. Ampullectomy-related mortality is exceptional, occurring in 0.3%[76].

Bleeding

The duodenal wall has a high vascularization. Bleeding can habitually stopped by hemostatic procedures (*e.g.*, adrenaline injection, APC, clipping)[88]. If substantial bleeding is expected then, biliary stent placement is useful to avoid cholagitis. If massive bleeding occurs, urgent arteriography is probably the best diagnostic and treatment option. In patients with a high risk of cardiovascular incidents aspirin may be continued; however, anti-coagulants agents should be discontinued.

Perforation

Perforation is usually retroperitoneal. Therefore, if perforation is suspected (endoscopic features, ongoing pain) a CT is more sensitive than simple radiology. Not all cases of perforation need surgical treatment, selected patients can be treated conservatively (intravenous antibiotics, gut rest)[6,14]. In anycase, a multi-disciplinary management is imperative to reach the best result.

ENDOSCOPIC OUTCOMES

The success rates for endoscopic resection of AA is high (range: 45%-92%), with recurrence rates of

Table 1 Recommended intervals for endoscopic surveillance after ampullectomy

	Surveillance
No residual polyp after the primary resection	3 mo later
If negative result for residual adenoma	1 yr later
Beyond this	every 3-5 yr
Patients with FAP	every 3 yr

FAP: Familial adenomatous polyposis.

0%-33%[9,89]. Intraductal adenoma growth had less favorable outcomes compared with adenomas without intraductal growth[15]. Predictors of success include: (1) lack of a genetic predisposition to adenoma formation (*e.g.*, FAP); (2) age > 48 years; (3) male sex; and (4) lesion size < 2.4 cm[70].

ENDOSCOPIC FOLLOW UP AND SURVEILLANCE

After ampullectomy patients should remain fasting for 4-12 h. Then, they are discharged home on a liquid diet and later continue with a normal diet. To reduce the risk of ductal lesion, the pancreatic stent should be removed in 2 wk.

Adenoma recurrence can occur in up to 25% of cases despite of complete removal during the index procedure[6,9,76]. In the absence of symptoms, surveillance endoscopy can be accomplished using a side-viewing duodendoscope without ERCP. Intervals change based on the histology and margin status of the resected lesion, history of FAP, patient age and comorbidities.

Recommended intervals (Table 1): (1) If there was no residual polyp after the primary resection: endoscopy 3 mo later; (2) If the result is negative for residual adenoma: surveillance 1 year later; (3) Beyond this, the yield of long-term surveillance in sporadic AA is unknown. We usually perform surveillance every 3-5 years; and (4) Given the risk for metachronous duodenal lesions, patients with FAP should undergo routine surveillance every 3 years.

CONCLUSION

Advances in endoscopy, EUS and ERCP have influenced the management to patients with ampullary lesions. Endoscopic ampullectomy has replaced surgical interventions for the treatment of AA without ductal extension. Endoscopic ampullectomy has lower morbidity and mortality rates than surgical approaches. Disadvantages include: difficult technique, few experienced endoscopists, several procedures to achieve total resection, moderate recurrence rates (30%), and, as with surgical ampullectomy, the need for postprocedure endoscopic surveillance. The best technique for endoscopic ampullectomy is subject to the adenoma size. *En bloc* resection is recomended for lesions confined to the papilla. Endoscopic ampullectomy is an effective and safe treatment for AA in experienced endoscopist but, the endoscopist must be alert to potential complications. Long-term follow-up information is required to clarify the appropiate surveillance interval for patients with sporadic AA.

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REVIEW

"How many times must a man look up before he can really see the sky?" Rheumatic cardiovascular disease in the era of multimodality imaging

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Abstract

Cardiovascular involvement in rheumatic diseases (RD) is the result of various pathophysiologic mechanisms including inflammation, accelerated atherosclerosis, myocardial ischemia, due to micro- or macro-vascular lesions and fibrosis. Noninvasive cardiovascular imaging, including echocardiography, nuclear techniques, cardiovascular computed tomography and cardiovascular magnetic resonance, represents the main diagnostic tool for early, non-invasive diagnosis of heart disease in RD. However, in the era of multimodality imaging and financial crisis there is an imperative need for rational use of imaging techniques in order to obtain the maximum benefit at the lowest possible cost for the health insurance system. The oligo-asymptomatic cardiovascular presentation and the high cardiovascular mortality of RD necessitate a reliable and reproducible diagnostic approach to catch early cardiovascular involvement. Echocardiography remains the routine cornerstone of cardiovascular evaluation. However, a normal echocardiogram can not always exclude cardiac involvement and/or identify heart disease acuity and pathophysiology. Therefore, cardiovascular magnetic resonance is a necessary adjunct complementary to echocardiography, especially in new onset heart failure and when there are conflicting data from clinical, electrocardiographic and echocardiographic evaluation of RD patients.

Key words: Echocardiography; Cardiovascular magnetic resonance; Nuclear imaging; Cardiovascular computed tomography; Myocardial perfusion-fibrosis; Coronary artery disease; Vasculitis; Rheumatic cardiovascular disease; Myocarditis

Core tip: The oligo-asymptomatic cardiovascular presentation and the high cardiovascular mortality of rheumatic diseases (RD) necessitate a reliable and

reproducible diagnostic approach to catch early cardiovascular involvement. Echocardiography, although being the cornerstone of cardiac evaluation, can not always exclude cardiac involvement and/or identify acuity and pathophysiology of cardiac lesions. Therefore, cardiovascular magnetic resonance is a necessary adjunct, complementary to echocardiography, especially in new onset heart failure and when there are conflicting data from clinical, electrocardiographic and echocardiographic evaluation of RD patients.

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INTRODUCTION

Cardiovascular involvement in rheumatic diseases (RD) is the result of various pathophysiologic mechanisms including systemic, myocardial, vascular inflammatory process, atherosclerosis, cardiac ischemia, due to micro and/or macrovascular lesions, abnormal coronary vasoreactivity and fibrosis[1,2]. RD patients may present with valvular, myocardial, pericardial, coronary artery or microvascular disease, vasculitis, systolic and diastolic heart failure, as well as pulmonary arterial hypertension. Symptoms of heart involvement in RD are usually subtle and underestimated, because they are attributed to the underlying systemic disease. The application of targeted treatment in RD led to a significant reduction of disease-associated mortality; however, the life expectancy of RD patients still remains lower, compared to general population[3], predominantly due to high incidence of cardiovascular disease[4-8].

RD with cardiovascular involvement include: (1) Rheumatoid arthritis and the spondyloarthropathies; (2) Systemic lupus erythematosus (SLE); (3) Systemic vasculitides; (4) Inflammatory myopathies; (5) Systemic sclerosis; (6) Mixed connective tissue diseases (MCTD); and (7) Sarcoidosis (SRC).

Non-invasive cardiovascular imaging, including echocardiography, nuclear techniques, cardiovascular computed tomography and cardiovascular magnetic resonance, represents useful diagnostic tool for early, non-invasive assessment of cardiac disease in RD. However, in the era of multimodality imaging and financial crisis there is an imperative need for rational use of the above mentioned techniques in order to obtain the maximum diagnostic benefit at the lowest possible cost for the health insurance system.

Applying the Bob Dylan's lyrics to cardiovascular evaluation of RD, it is clear that a careful and cost-effective evaluation of all available imaging techniques is needed, before to achieve the best diagnostic approach. The aim of this review is to present the value of various imaging techniques and propose an efficient diagnostic algorithm for early detection of cardiovascular involvement in RD.

NON-INVASIVE IMAGING TECHNIQUES

Echocardiography

Currently, the most commonly noninvasive, imaging technique, used in cardiovascular imaging, is echocardiography, due to high availability, portability, low cost, lack of radiation and high expertise among cardiologists. Transthoracic echocardiography (TE) allows an accurate evaluation of valvular morphology and function, assessment of pericardium and abnormalities of ventricular wall motion; by adding Doppler analysis, valuable information about left ventricular diastolic function, valvular flow and pulmonary pressure can be also obtained. Recently, it was documented that the definition of pulmonary hypertension obtained by echocardiography is useful to predict the 6-year mortality in SLE[9]. In another study evaluating patients with antiphospholid syndrome (APS), pulmonary hypertension was the most common finding in APS and was associated with thromboembolic disease; in contrary left ventricular disease and cardiac thrombi were rare[10]. Furthermore, in APS and SLE (with or without aPL), SLE/APS and disease duration were independent predictors for valvular disease progression and ventricular diastolic dysfunction in a 10-year follow-up echocardiographic evaluation[11]. In a meta-analysis, the presence of aPL in SLE was associated with high risk for heart valvular disease, including Libman-Sacks endocarditis. Therefore, systematic echocardiography evaluation in SLE with aPL should be always scheduled[12]. Echocardiography has been successfully used in both antiphospholipid syndrome[11,12] and asymptomatic patients with juvenile-onset SLE, who presented evidence of declining ventricular diastolic function with time[13]. Rexhepaj *et al*[14] found significant differences in early diastolic flow velocity (E), atrial flow velocity (A) and E/A ratios in rheumatoid arthritis (RA) compared with normals, suggesting that a subclinical lesion of left and right ventricular function is present in RA patients, although left ventricular parameters were still normal. Improvement of cardiac function was also shown by conventional echocardiography in RA after treatment with infliximab[15].

Another application is transthoracic dipyridamole stress echocardiography and coronary flow reserve (CFR) evaluation. CFR is assessed in the distal left anterior descending coronary artery expressed as ratio between peak diastolic velocity during stress and at baseline. It is an extremely sensitive marker (> 90%) for coronary artery disease (CAD)[16,17] and, if it was considered together with regional wall motion abnormalities, is even more specific[18]. A CFR < 2 is a very accurate index to predict the presence of CAD[17]. If coronary arteries are normal, CFR abnormalities show impairment of coronary microcirculation, as in

arterial hypertension with or without left ventricular hypertrophy, diabetes mellitus, hypercholesterolemia, syndrome X, hypertrophic cardiomyopathy and other diseases[19]. A reduced CFR reflects bad CAD prognosis[20]. Finally, we should emphasize that not only the binary (normal-abnormal) response in CFR, but also the continuous spectrum of CFR values is a strong independent predictor in known or suspected CAD[21]. Hirata *et al*[22] found a serious reduction of CFR in premenopausal SLE women compared with matched controls, due to microvascular disease that leads to decreased vasodilation during pharmacological stress. Turiel *et al*[23] detected a significant impairment of CFR in early RA without any anti-rheumatic therapy and disease duration < 1 year. The reduced CFR in the absence of wall motion abnormalities indicated abnormalities of coronary microcirculation, due to endothelial dysfunction.

Furthermore, exercise echocardiography (EE) using dobutamine was proven of great value for the evaluation of myocardial ischemia in RD. In a study by Saghir *et al*[24], RA was associated with a 2-fold higher risk for cardiac ischemia on EE and the risk was depending on RA disease duration; mortality rate was also higher in RA with ischemic EE study[24]. Furthermore, asymptomatic RA patients may present cardiac ischaemia at similar levels to DM patients but with low prevalence of obstructive coronary artery lesions and higher incidence of microvascular disease, due to increased inflammatory response[25].

Tissue Doppler imaging (TDI) is a new echocardiographic technique that allows the measurement of myocardial velocities and myocardial deformation. It is limited by the angle used, because only deformation along the ultrasound beam can be used from velocities evaluation; however, myocardium presents simultaneous deformation in 3 dimensions[24]. Birdane *et al*[26] demonstrated that RA patients had a significant impairment of TDI biventricular diastolic function compared with controls that was depended on age and steroids treatment. To overcome TDI limitations, speckle tracking analysis has been applied to evaluate myocardial strain along the longitudinal, circumferential and radial axes[27]. Recently, it was demonstrated that interleukin-1 inhibition contributes to a greater amelioration in endothelial, coronary and aortic function in addition to left ventricular myocardial deformation and twisting in RA patients with CAD than in those without[28]. Additionally, global longitudinal LV/RV strain was reduced in RA patients compared with controls and strain abnormalities were correlated with RA disease severity[29]. Furthermore, 3D-speckle tracking is a new method to detect early abnormalities in SLE patients with normal LV systolic function assessed by 2D echocardiography[30].

Another application of echocardiography which is very useful in systemic autoimmune diseases is transesophageal echocardiography (TOE). TOE is more sensitive compared with TE for the detection of valvular lesions and cardiac masses[31]. Turiel *et al*[32] detected a high prevalence (61%) of valvular abnormalities or vegetations as potential embolic causes using TOE in 56 patients with primary APS. Recently, the development of 3D TOE allows cross-sectional visualization of the mitral, aortic and tricuspid valves, improving the diagnostic sensitivity compared to traditional 2D imaging[33]. Its superiority over 2D echocardiography includes more accurate and reproducible calculation of LV volumes, mass and ejection fraction, more accurate identification of wall motion changes, more reliable evaluation of right ventricle and better assessment of valvular and subvalvular abnormalities[34].

Echocardiography represents the most versatile and popular cardiac imaging modality easily applicable in any patient from outpatient clinic to intensive care unit; however, it carries some limitations. It is operator dependent, has the limitation of the acoustic window, cannot perform detailed tissue characterization and cannot define the type of tissue lesions in patients with preserved diastolic or systolic function[35].

Nuclear techniques

Single-photon emission computed tomography: It is the most widely used nuclear technique to evaluate stress/rest cardiac perfusion. Maximal exercise or pharmacological stress can be used as a stressing factor and diffusible radiotracers injected during the peak stress allow the detection of myocardial stress perfusion defects[36]. Myocardial blood flow during stress increases about 3 to 5 fold compared with the values during rest. If there is a significant coronary stenosis, myocardial perfusion will not increase adequately in the territory supplied by the stenotic artery conducting to perfusion defect. The currently used single-photon emission computed tomography (SPECT) radiotracers are characterized by a myocardial uptake proportional to blood flow[37]. SPECT is considered as a very sensitive technique for the detection of myocardial ischemia[38]; however, its specificity is relatively lower[39], mainly due to soft-tissue attenuation artifacts. Additional disadvantages of SPECT include high cost and the use of radioactive materials[40].

Positron emission tomography: It has higher spatial resolution compared with SPECT and provides absolute quantitative information about the physiologic parameters of myocardium; moreover, it has high sensitivity and specificity for assessment of myocardial ischemia compared with SPECT. Recently, positron emission tomography (PET) with flurpiridaz F 18 was proven safe and superior to SPECT, due to better image quality, higher certainty during interpretation and generally better CAD diagnosis[41].

Myocardial perfusion by nuclear techniques is a useful tool for the detection of subclinical CAD in RD[42]. Silent myocardial infarction has been also diagnosed by myocardial perfusion SPECT in SLE[43]. Additionally, abnormal perfusion was identified in asymptomatic,

low risk for CAD in SLE patients using a technetium-99m sestamibi[44]. Finally, in SLE patients with cardiac symptoms an abnormal glucose metabolism of the myocardium was detected, shown as a pathological 18FDG scan, whereas perfusion appeared normal (reversed mismatch)[45]. In inflammatory myopathies, Technetium-99m pyrophosphate (99mTc-PYP) and gallium-67 scans had similar sensitivity, specificity and accuracy in the detection of skeletal muscle disease, compared with serum enzymes (70%, 100% and 80%, respectively). Compared with clinical parameters, 99mTc-PYP presented 70% and 67Ga 65% accuracy. Abnormal PYP and 67Ga cardiac uptake was observed in 57% and 15% of patients, respectively[46].

However, nuclear imaging techniques have the disadvantages of high cost, radiation, inability to perform tissue characterization and low spatial resolution, not allowing the assessment of subepicardial, intramyocardial or subendocardial fibrotic lesions, frequently found in RD[33].

Multislice computed tomography

Coronary artery calcification (CAC) occurs due to atherosclerotic process and reflects the total coronary atherosclerotic burden[47]. The Agatston coronary calcium score identifies the extent of calcification in the coronary arteries[48]. Electron-beam computed tomography (EBCT) is a very sensitive technique able to detect small depositions of calcium in the coronary arteries. The radiation dose during an EBCT is considerably lower compared with X ray coronary angiography[49]. Recently published studies, using multislice computed tomography (CT) with iodinate contrast agents to visualize the coronary artery lumen, demonstrated high accuracy in the early diagnosis of CAD[50]. This technique plays a diagnostic role not only for the detection of significant coronary artery stenosis, but also for tissue characterization of the atherosclerotic plaque. Moreover, it allows coronary calcium assessment along the coronary arteries[51]. CT requires iodinated contrast agents, which could provoke symptoms of intolerance and/or renal impairment. Furthermore, during the CT examination patients undergo ionizing radiation exposure. The prevalence of CAD using CTA in asymptomatic high-risk patients is high. If coronary artery calcium score is zero, it can not exclude CAD; however, a normal CTA is extremely accurate to exclude CAD. Total coronary plaque burden, even if only one segment is involved, are associated with high risk for cardiac events[52]. Finally, according to the recently published CONFIRM study, coronary CT angiography has incremental prognostic value for prediction of mortality and non-fatal myocardial infarction in asymptomatic patients with moderately high coronary artery calcium score (CACS), but not in lower or higher CACS[53].

Using CT, it was documented that SLE patients had significantly higher prevalence and/or extent of arterial calcification, compared with matched controls[54] and the disease activity was a potentially modifiable risk factor[55]. Finally, another CT study demonstrated that the calcification of cardiac valves is more prevalent in RA and SLE, compared with controls. The presence of mitral valve, but not aortic valve calcifications, independently predicted premature atherosclerosis in RA and SLE[56]. Furthermore, RA patients without CAD had higher prevalence and severity of all types of coronary plaque. Residual disease activity associates with higher incidence of non-calcified and mixed plaques contributing to future cardiac events[57]. In another CT study, it was also documented that coronary atherosclerosis was not uncommon in asymptomatic SSc patients[58]. Finally, CT scan is the technique of choice for assessment of pulmonary embolism and pulmonary hypertension secondary due to recurrent pulmonary emboli[59].

Cardiovascular magnetic resonance

Cardiovascular magnetic resonance (CMR) is a non-invasive, nonradiating, operator independent technique that can offer reliable and reproducible information about myocardial function, inflammation, perfusion, fibrosis and heart disease acuity; additionally, vascular disease acuity and vascular inflammation and/or stenosis can be also assessed.

Table 1 summarises the most frequent findings, advantages and disadvantages of each methodology.

The evaluation of rheumatic diseases by CMR can offer: (1) Angiography, imaging of vessel wall and cardiac evaluation (function, oedema, early, late gadolinium enhancement and stress CMR) in vasculitis. Techniques for angiography include both contrast-enhanced MR angiography (CE-MRA) as well as non-contrast methods. Pre-contrast T1W and T2W dark blood imaging but also post-gadolinium T1W imaging can reveal presence of inflammation, even when the disease is clinically under remission[60]; (2) Function, oedema, early, late gadolinium enhancement and stress CMR for RA, SLE, SSc and MTCD. Evidence of myocardial inflammation and/or fibrosis can be identified by STIR T2, early and late gadolinium enhancement, even if the rheumatic disease is under remission[61,62]; (3) Additionally, it is the gatekeeper for differential diagnosis between various types of scar: scar due to CAD that should motivate coronary artery evaluation (subendocardial or transmural scar following the distribution of coronary arteries in CAD) and scar due to inflammation or vasculitis (subepicardial or intramural scar not following the distribution of coronary arteries in inflammation and diffuse subendocardial fibrosis in case of diffuse subendocardial vasculitis)[61-63]; (4) Function, oedema, early and late gadolinium enhancement in inflammatory myopathies using SSFP, STIR T2, early and late gadolinium enhancement, even if the disease is under remission[64,65]; (5) Carotid angiography and vessel wall imaging in RA and SLE[60]; (6) Coronary angiography, oedema, early, late gadolinium enhancement, stress CMR and scar detection for Kawasaki disease[66]; and (7) Assessment of PAH includes information about

Table 1 Clinical findings, advantages and disadvantages of each technique

Noninvasive imaging techniques	Evaluation of	Advantages	Disadvantages
Rest echocardiography	Cardiac valves	Cheap	Operator dependent
	Pericardium	Widely available	Limitation due to poor acoustic window
	Ventricular function	Bedside	No tissue characterization
	Wall motion	No radiation	
	Pulmonary pressure		
Tissue doppler imaging	Measurement of myocardial velocities	The same as in rest echocardiography	Limited by angle-dependency
Stress echocardiography	CFR in LAD	The same as in rest echocardiography	The same as in rest echocardiography
	Myocardial ischemia		
Transesophageal echocardiography	Valvular lesions	The same as in rest echocardiography	The same as in rest echocardiography
	Intracardiac masses		Semi-invasive
SPECT	Myocardial ischemia	Widely available	Radiation
	Ventricular function	Reasonable sensitive	High cost
		Not very specific	Low spatial resolution
PET	Myocardial ischemia	Very sensitive	Radiation
	Ventricular function	Very specific	High cost
			Low spatial resolution
			Not widely available
CT	Great vessels	Fast	Radiation
	Coronary arteries/grafts	Widely available	High cost
			Iodinated contrast agent
CMR	Ventricular function	Highly reproducible	Not widely available
	Inflammation	Operator independent	High Cost
	Perfusion	No radiation	Claustrophobia
	Fibrosis	Tissue characterisation	Non MRI compatible devices can not be scanned
	Heart disease acuity	High spatial resolution	Low temporal resolution
	Vascular disease acuity		
	Vascular inflammation and/or stenosis		

SPECT: Single-photon emission computed tomography; PET: Positron emission tomography; CMR: Cardiovascular magnetic resonance; CFR: Coronary flow reserve; CT: Computed tomography; LAD: Left anterior descending.

right ventricular (RV) mass index, RV volumes-ejection fraction, late gadolinium enhancement (LGE) and phase contrast imaging, including average velocity (cm/s), retrograde flow (L/min) and percentage retrograde flow (%)[67]. These indexes have been shown to be prognostic of long-term outcomes. LGE at ventricular insertion points in PAH is due to altered intraventricular septal motion and not to elevated RV pressure or remodelling[68].

TOWARDS AN ALGORITHM ABOUT THE APPLICATION OF NONINVASIVE CARDIOVASCULAR IMAGING IN RHEUMATIC DISEASES

The first line and cornerstone of routine cardiac assessment in RD is echocardiography. However, it is unable to detect cardiac disease acuity, myocardial or vascular inflammation and scar in cases with normal LV function[69]. Nuclear techniques are also unable to detect small perfusion defects, commonly found in RD, due to low spatial resolution, to identify disease acuity and the exact location of the lesion (subendocardial, transmural or subepicardial) and to further guide risk stratification[70]. CT coronary angiography cannot be included in the routine assessment of cardiac involvement in RD, because it cannot answer all the relevant queries, raised in these diseases. Furthermore, high cost, the need of repetitive radiation in both nuclear techniques and CT and the use of iodinated contrast agents in CT constitute serious limitations for its routine use in diagnosis and follow up.

CMR is a non-invasive, nonradiating, highly reproducible technique, capable to answer queries about cardiac disease acuity, etiology of cardiac lesion, necessity for cardiac catheterization and persistence of myocardial involvement, although the systemic disease seems quiescent[69].

CONCLUSION

The oligo-asymptomatic cardiovascular presentation and the high cardiovascular mortality of RD necessitate a reliable and reproducible diagnostic approach to catch early cardiovascular involvement. Echocardiography remains the routine cornerstone of cardiac evaluation. However, a normal echocardiogram cannot always exclude cardiac involvement and/or identify acuity and pathophysiology of cardiac lesions. Therefore, CMR is a necessary adjunct, complementary to echocardiography, especially in new onset heart failure and when there are conflicting data from clinical, electrocardiographic and echocardiographic evaluation of RD patients.

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Lamb's head: The model for novice education in endoscopic sinus surgery

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Abstract

Structured training in endonasal endoscopic sinus surgery (EESS) and skull base surgery is essential considering serious potential complications. We have developed a detailed concept on training these surgical skills on the lamb's head. This simple and extremely cheap model offers the possibility of training even more demanding and advanced procedures in human endonasal endoscopic surgery such as: frontal sinus surgery, orbital decompression, cerebrospinal fluid-leak repair followed also by the naso-septal flap, *etc.* Unfortunately, the sphenoid sinus surgery cannot be practiced since quadrupeds do not have this sinus. Still, despite this anatomical limitation, it seems that the lamb's head can be very useful even for the surgeons already practicing EESS, but in a limited edition because of a lack of the experience and dexterity. Only after gaining the essential surgical skills of this demanding field it makes sense to go for the expensive trainings on the human cadaveric model.

Key words: Endonasal; Endoscopic; Sinus surgery; Skull base; Learning; Training; Lamb's head

Core tip: Structured training in endonasal endoscopic sinus surgery (EESS) and skull base surgery is essential considering serious potential complications. We have developed a detailed concept on training these surgical skills on the lamb's head. This simple and extremely cheap model offers the possibility of training even more demanding and advanced procedures in human EESS such as: frontal sinus surgery, orbital decompression, cerebrospinal fluid-leak repair followed also by the naso-septal flap, *etc.* Unfortunately, the sphenoid sinus surgery cannot be practiced since quadrupeds

do not have this sinus. Still, despite this morphological limitation, it seems that the lamb's head can be very useful model.

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INTRODUCTION

Endonasal endoscopic sinus surgery (EESS) procedures are one of the most common surgical procedures performed in nowadays otorhinolaryngology. They are considered the gold-standard treatment for several entities regarding the paranasal sinuses and nasal cavity[1,2]. The orientation within the anatomic structures and a proper use of the surgical instruments during the procedures is challenging for the inexperienced surgeon, owing to the complexity of the intranasal anatomy and the intimate relation with noble structures, such as the brain, the carotid arteries, the eye-ball, orbit and the optic nerve itself[1-4]. Nowadays, the training of the novices in endonasal sinus and skull base surgery is conducted in the operating room, upon the real patients, under the surveillance of more experienced surgeons[1,2]. The complication rate in endonasal sinus and skull base surgery may vary from 5% to 8%[2]. The low and flat learning curve of the particular novices may increase the risk of serious complications. To prevent additional problems to both novices and their supervisors as well as to protect patients, all activities that objectively can help to over-bridge the gaps in theoretical and practical expertise should be employed. One of these activities undoubtedly is the lamb's head dissection training according to our model.

In terms of that, a comprehensive booklet on this matter has been written by our team and published so far in Croatian, English, Italian and Russian language by Karl Storz GmbH (Germany)[5]. In collaboration with the same manufacturer we have produced also an attractive DVD on complete dissection in a "step-by-step" manner.

LAMB'S HEAD ANATOMY

The first distinctive feature that arises when entering the lamb's nose is an abundant similarity to the human nasal cavity. Lamb's septum does not have vomer at all, thus a typical triangular lack of septum can be seen endoscopicaly in the deepest septal regions.

The inferior turbinate could be most confusing detail at the beginning since it resembles very much the middle turbinate in man. In veterinary terminology it is named *concha ventralis,* meaning anterior turbinate. According to the veterinary anatomical nomenclature, the term "middle turbinate" (*concha nasalis media*) in lamb is used to denominate a structure that is located much deeper in the lamb's nose, and can be clearly seen only if almost all of the inferior turbinate has been removed. The lamb's inferior turbinate has two main portions: the superior one, named *pars dorsalis* and the inferior one, named *pars ventralis.*

The nasal septum is straight as in all other quadrupeds. The maxillary sinus consist of two "sub-sinuses" since a perpendicular crest, arising from the bottom of this sinus, divides it in a laterally positioned, spacious cavity, so called *maxillary sinus proper*, and medially positioned, poky sinus named *palatinal sinus*. The superior, free edge of the crest that divides maxillary cavity into two sinuses is characterized by the course of the infraorbital nerve within its bone. The formation of the middle antrostomy is easy, simple, instructive and motivating for the next steps of the dissection. Posterior wall of both palatal sinus and maxillary sinus proper is at the same time the anterior orbital wall thus making an endoscopic approach to the orbital decompression relatively simple. The frontal sinus consists of queue of chambers positioned semicircular in the frontal bone thus forming a structure that resembles very much a crown. Frontal sinus is also relatively easy to approach since the surgeon has a reliable signpost: the superior turbinate gradually, as getting deeper and deeper with the instruments and endoscope, gets a tube-like form. At the bottom of this tube the surgeon finds himself in the first, most anteriorly positioned, so called supraorbital frontal sinus cell. The sphenoid sinus surgery can't be practiced in lamb's head model since quadrupeds, because of the lack of the skull base angulation (*Huxley's angle*) do not have this sinus.

LAMB'S HEAD PREPARATION

The lamb heads are purchased fresh at the butcher's shop (approximate price is about two US$) and the muzzle is always cut off (cartilaginous part) as to make the entrance to the nasal cavities much easier and practical. After that, the heads are washed under the tap water and then put into a tree-liters bowl of water containing three tablespoons of alcoholic vinegar. After 24 h of soaking the heads are usually left to drain in the sink for 30 min, than wiped with a clean cloth and finally frozen at -18 ℃ until the moment they will be used for the dissection. On the day of dissection the heads are unfrozen, washed and wiped. Heads prepared in this manner are far more easy to use, without an excess of grease and fluids. For the dissection purposes the heads are mounted in a special Lamb's Head Holder (Karl Storz GmbH) (Figure 1).

DISSECTION TECHNIQUE

The dissection usually consists of ten classical steps. Step 1 concerns to the very simple task: removal of the inferior turbinate; Step 2 concerns to the clear

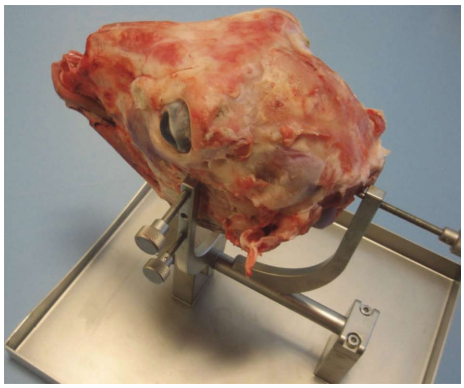

Figure 1 The head holder. Six sideward-screws serve to fix the lamb's head in desired position while dissecting.

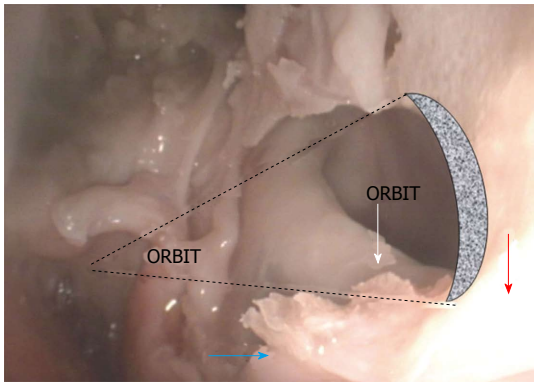

Figure 2 Endoscopic view to the left maxillary complex. The perpendicular crest (white arrow) divides the cavity within the maxilla into maxillary sinus proper (red arrow) and palatine sinus (blue arrow).

presentation of the adenoids and Eustachian tube openings bilaterally, while Step 3 regards to the clear presentation of the middle turbinate, uncinectomy and middle antrostomy followed by as clear as possible presentation of the perpendicular crest within its cavity that divides it into the maxillary sinus proper (lateral compartment), and palatine sinus (medial compartment) (Figure 2). The superior edge of this crest contains the infraorbital nerve canal; Step 4 means the identification of the posterior wall of palatine sinus which is, at the same time, the medial half of the anterior orbital wall, followed by alignment with its lateral half, *i.e*., posterior wall of the maxillary sinus proper; Step 5 is performed in sense of the endonasal endoscopic orbital decompression; Step 6 belongs to the ethmoidectomy, whereas Step 7 mean the formation of the artificial skull base defect, presentation of dura, followed by insertion of the artificial patch in the underlay manner; Step 8 means an elevation and adequate positioning of the nasoseptal flap to be used to cover the closed skull base defect in an overlay manner. The next, 9th Step, belongs to the removal of the tube-like formation, so called dorsal turbinate (plica recta), positioned at the anterior part of the roof of the

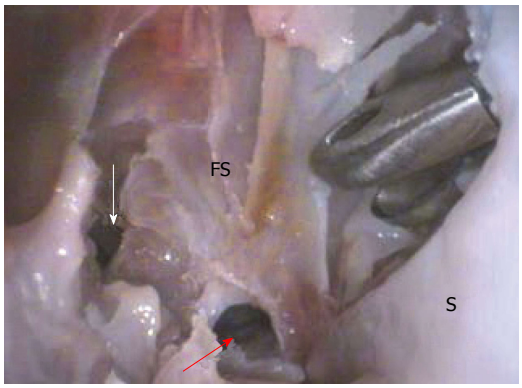

Figure 3 Endoscopic 30° view at the region of the bottom of the frontal sinus. The tip of the Kerisson's punch juts from the left nasal cavity through the artificially made septal (S) defect. Red and white arrows indicate the frontal sinus lateral and anterior cells. FS: Frontal sinus.

nasal cavity, which transforms gradually, getting more posterior, into dorsal turbinate and leads directly to the opening of the supraorbital frontal sinus chamber first. Once opened, this cell leads the surgeon to the more anteriorly and medially positioned frontal sinus cells. Finally, the Step 10 concerns to the removal of the most superior part of the nasal septum as to make the bottom of the frontal sinus visible from both sides and thus bilaterally approachable. Afterwards, the same procedure as described in Step 9 is repeated on the opposite side in order to open the contra lateral frontal sinus chambers. Finally one has to drill out the bony bottom of the frontal sinus thus connecting the previously opened cells in one, large cavity - Draf Ⅲ procedure (Figure 3).

DISCUSSION

Safe endonasal endoscopic sinus and skull base surgery requires a good and reliable knowledge on surgical anatomy and, at the same time, the safe usage of the endoscopes and instruments. Many institutions around the world insist on trainings performed upon the human cadaveric model before the surgeon undertakes his/her first surgery on a real patient[6-8]. Training courses and workshops in EESS provide a well structured supervised approach to endonasal anatomy and technical training. Unfortunately, these trainings can be achieved once or, at the most, twice during the particular novice's education because of very high registration fees for the courses. Even more, they do not offer the calm that the novice, confused and scared from all sides, can't get. Such courses, regardless of the fact that almost always are perfectly organized, have the disadvantage, not only of being such expensive, but also of being dependent on the strict medico-legal regulations regarding the question of collecting, storage and disposal of the human cadaveric heads which, nowadays, represents a rapidly growing problem in the increasing number of countries all over. Artificial models of human heads were also designed and available for training[9] but are

highly priced. Furthermore, endoscopic sinus surgery simulators have also been introduced as teaching tools for EESS[1,10,11].

However, in our opinion, the initial goal for the novices in the field of endoscopic endonasal sinus surgery is to gain the surgical skills and to become familiar with the complexity of simultaneous bimanual work in operating field, distant from that what they actually see on the monitor during their work. So, the trainees should be firstly trained on the animal models as to become more skilled with the endoscopes of various angles, orientation and understanding the surgical space and field, and to get skilled in the use of the instruments. The study of human anatomy on the cadaveric dissections should be only the second step in any case. In this way the learning curve for EESS gets more attractive thus giving the novices, *i.e.*, trainees more confidence in operating area and better understanding of anatomical details they are about to dissect. Animal model of a comparable sino-nasal appearance to human seems to be the logical choice for the initial training of surgical skills.

Paying a great respect to Gardiner's introduction of a sheep model for training in EESS[12], we have developed a detailed study for training on the lamb's head, combining radiology findings, frozen three-axis sections and a meticulous research on lamb's sino-nasal surgical anatomy as to facilitate the first steps in EESS for all those who approach this field[5].

This was the result of long lasting attempts to find a low-cost and suitable animal model that will fit two most important demands: (1) high degree of the anatomical similarity to human sino-nasal anatomy; and (2) the appropriate dimensions of the sino-nasal unit that will allow the easy use of the standard endoscopic sinus surgery instruments otherwise used in human medicine. We have tried with dogs, pigs, sheep and goats, but, at the end, we found everything we needed with the lamb's head[13]. Some years ago we also developed a special head holder for the lamb's head, produced by Karl Storz, Germany. The lamb's head as a simple and extremely cheap model offers the possibility of training even more demanding and advanced procedures in human endonasal endoscopic surgery such as endoscopic endonasal orbital decompression, Draf Ⅲ procedure or cerebrospinal fluid leak repair, including the naso-septal flap[14]. Dacryocystorhinostomy can't be performed since the lamb does not have the lacrimal sac at all[4]. The sphenoid sinus surgery can't be practiced either, since quadrupeds do not have this sinus because of the natural lack of the skull base angulation. Still, despite these two morphological limitations, it seems that the lamb's head model can be very useful also for the surgeons that want to amend their technique and take it to a higher level. In terms of that, it was mandatory to have also the navigational system for the lamb's head and it was built by Karl Storz GmbH as well.

Regarding maxillary sinus, it's amazingly easy to perform middle antrostomy Frontal sinus surgery resembling Draf 1, 2 and 3 procedures can be trained with the ease and calm as well.

In our opinion the lamb's head is an effective, extremely cheap and user-friendly animal model for learning and training the endonasal endoscopic sinus and skull base surgery techniques.

CONCLUSION

We have developed a practical and detailed program for the training of surgical techniques in EESS on the lamb's head, combining radiology findings, frozen three-axis sections and a meticulous research on lamb's sinonasal surgical anatomy. Lamb's head proved to be an excellent model with comparable anatomy to that in humans and thus very appropriate to practice the usual EESS techniques with ease using the standard EESS instruments.

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MINIREVIEWS

Refractory chronic cough due to gastroesophageal reflux: Definition, mechanism and management

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Abstract

Refractory chronic cough due to gastroesophageal reflux is a troublesome condition unresponsive to the standard medical anti-reflux therapy. Its underlying mechanisms may include incomplete acid suppression, non-acid reflux, transient lower esophageal sphincter relaxations and esophageal hypersensitivity. The diagnosis of this disorder depends on both the findings of multi-channel intraluminal impedance-pH monitoring and the subsequent intensified anti-reflux therapy. The strategies of pharmacological treatment for refractory chronic cough due to reflux include the optimization of proton pump inhibitors and add-on therapies with histamine H_2 receptor antagonists, baclofen and gabapentin. However, the further study is needed to satisfy its management.

Key words: Esophageal pH monitoring; Chronic cough; Anti-reflux therapy; Refractory cough; Gastroesophageal reflux

Core tip: Refractory cough due to reflux can be defined as a reflux-induced cough resistant to standard medical anti-reflux treatment but responsive to the subsequent intensified anti-reflux therapy. It may be associated with the residual acid or non-acid reflux, transient lower esophageal sphincter relaxations and esophageal hypersensitivity. The definite diagnosis of the disorder depends on the positive findings of multi-channel intraluminal impedance-pH monitoring as well as favorable response to the intensified anti-reflux therapy. The current therapeutic strategies include the complete acid suppression and add-on uses of baclofen or gabapentin.

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INTRODUCTION

Gastroesophageal reflux-induced chronic cough (GERC) is a special form of gastroesophageal reflux disease with predominant cough symptom[1] and along with cough variant asthma, upper airway cough syndrome or eosinophilic bronchitis, is considered as a common cause of chronic cough[2,3]. Like gastroesophageal reflux disease, proton pump inhibitors (PPIs) alone or in combination with prokinetic agents are a standard medical therapy for GERC and can resolve the cough in most patients[1]. However, a small percentage of patients with GERC are resistant to the standard anti-reflux treatment and this condition is also defined as refractory GERC[4]. This review summarizes our understanding about the definition, mechanism and management of refractory GERC.

DEFINITION OF REFRACTORY GERC

How to define refractory GERC remains to be controversial. There is no consensus on the refractory gastroesophageal reflux disease which GERC can refer to. The generally accepted definition of refractory gastroesophageal reflux disease is the persistent classical reflux-related symptoms such as regurgitation and heartburn despite the treatment with PPIs twice daily for at least 4-8 wk[5]. Recently, Sifrim *et al*[6] proposed that refractory gastroesophageal reflux disease should be defined as the condition in which symptoms (heartburn and/or regurgitation) are not responsive to a stable double dose of PPIs during a treatment period of at least 12 wk and patients continue to report troublesome symptoms while "on" PPIs at least thrice weekly for the last 3 mo[6]. As one of extraesophageal symptoms, cough can be caused by many diseases other than GERC. A cause-effect association between reflux and cough is more difficult to establish than regurgitation and heartburn, and too long-lasting trial with PPIs may delay the diagnosis and treatment of the other etiologies of chronic cough. Therefore, we have defined refractory GERC as a condition of chronic cough with the objective evidence of abnormal reflux as demonstrated by multichannel intraluminal impedance-pH monitoring (MII-pH), and resistant to a 8-wk standard medical anti-reflux treatment but responsive to the subsequent intensified anti-reflux therapy[4,7]. This definition is consistent with the principles recommended in several guidelines for the management of chronic cough[1,8] as well as the generally accepted definition of refractory gastroesophageal reflux disease[5].

The exact prevalence of refractory GERC is still unclear. It is estimated that 10%-40% patients with gastroesophageal reflux disease do not or only partially respond to the standard dose of PPIs[9]. Unlike erosive esophagitis, nonerosive reflux disease has basically normal esophageal mucosa under an endoscope and normal or slightly abnormal esophageal acid exposure as indicated by MII-pH, accounts for 70% of gastroesophageal reflux disease and is poorly responsive to PPIs treatment[10,11]. Therefore, nonerosive reflux disease is responsible for the majority of refractory gastroesophageal reflux disease. Our preliminary results have shown that refractory GERC accounts for about one third of GERC[12], and is comparable with the prevalence of refractory gastroesophageal reflux disease.

MECHANISMS OF REFRACTORY GERC

It is well known that GERC may be caused by microaspiration of the refluxate into the airways (reflux hypothesis) and esophageal-tracheobronchial reflexes mediated by the afferent nerves in the distal esophagus (reflex hypothesis)[1]. However, the mechanisms underlying the refractory GERC is poorly understood. It may be associated with the incomplete acid suppression, non-acid reflux, transient lower esophageal sphincter relaxations (TLESRs) and esophageal hypersensitivity.

INCOMPLETE ACID SUPPRESSION

Incomplete acid suppression has been documented in patients with persistent symptoms despite the therapy with PPIs at a standard dose. Several studies have shown 4%-17% patients presented with abnormal acid reflux[13,14] and 7%-11% patients had a positive symptom index[15,16] as revealed by 24-h esophageal pH monitoring when they were "on" PPIs. The residual acid reflux can continue to elicit cough through microaspiration or esophageal-tracheobronchial reflex[1]. In addition to poor compliance such as not taking PPIs in time or at the suitable time, ineffective acid suppression may also be related to the difference in the responsiveness to therapy among patients. For example, the rapid metabolism of PPIs in some patients may result in that the high serum PPIs level can not be achieved for the adequate acid suppression[17]. Nocturnal acid breakthrough, a phenomenon of gastric pH below 4 for at least 1 h during the night, was also suggested as a cause of the failure to PPIs treatment by promoting gastroesophageal reflux during sleep[18]. However, accumulating evidence does not support a significant role of nocturnal acid breakthrough in the failure of PPIs treatment.

NON-ACID REFLUX

Non-acid reflux, an important constituent of reflux, includes weakly acidic (refluxate pH = 4-7) and weakly alkaline (refluxate pH > 7) reflux, with 95% belonging to weakly acidic reflux and only 5% belonging to weakly alkaline reflux[19]. Non-acid reflux accounts for 50% and 95% of reflux in the patients with gastroesophageal reflux disease "off" and "on" PPIs, respectively[20]. It is reported that cough-related reflux consists of acid (65%), weakly acidic (29%) and weakly alkaline (6%) reflux in the GERC patients "off" acid suppressive therapy[21]. In contrast, reflux-related cough is caused

by weakly acidic (74%), weakly alkaline (17%) and acid (4%) reflux in patients with refractory GERC[22]. The increase in weakly acidic reflux episode may derive from the relative increase in the percentage of original weakly acidic reflux due to the inhibition of acid reflux or in a considerable part, the transition of the original acid reflux due to pH value shift in the refluxates after PPIs treatment. The cough induced by weakly acidic reflux may be associated with esophageal distension due to increased reflux volume, persistent impaired mucosal integrity and esophageal hypersensitivity[23].

TLESRS

TLESRs refer to the spontaneous (not preceded by a swallow) relaxations of the lower esophageal sphincter lasting 10-60 s[24], which is a vagally mediated event induced by the volume distension of the stomach. Physiologically, it plays a role in venting air from the stomach after meals and also represents a main mechanism underlying all types of reflux[25]. In patients with established gastroesophageal reflux disease, TLESRs have a high prevalence and is two times more likely to be related to the reflux[26]. In general, PPIs can reduce the acidity and volume of refluxate in the esophagus, but have no ability to rectify the dysfunction of the lower esophageal sphincter and decrease reflux episodes.

ESOPHAGEAL HYPERSENSITIVITY

Esophageal hypersensitivity is defined as an exaggerated response of esophageal mucosa to normal or subthreshold stimuli and involved in the pathogenesis of GERC. It is unclear whether esophageal sensitivity in refractory GERC is higher than naive GERC. However, several lines of evidence have demonstrated that patients with nonerosive reflux disease are more sensitive to intraesophageal acid infusion, balloon distension and electrical stimulation than patients with erosive esophagitis[27,28].

The function of peripheral sensory terminals may be modified by inflammatory mediators released from the injured and inflammatory esophageal mocosa caused by reflux. Consequently, the transduction threshold is decreased in the primary sensory afferents, resulting in hypersensitivity at the site of injury or inflammation, and a heightened response to subthreshold or innocuous chemical, mechanical and electrical stimuli. It has been shown that the expression of acid-sensing receptors and transient receptor potential vanilloid 1 are up-regulated in the esophageal mucosa of patients with gastroesophageal reflux disease[28]. Patients with refractory GERC have the dilated intercellular spaces in the esophageal epithelium due to repeated exposure to acid and enzymes, which permits the penetration of some noxious or sensitizing substances through the epithelial barrier, exposes and activates subepithelial nerves, and prompts the transduction of acid signals from the peripheral afferents to cough center[29]. Once central sensitization is established, it can continue to potentiate cough even though the initial peripheral stimulus is discontinued.

DIAGNOSTIC APPROACH

According to the algorithms recommended in several guidelines for the management of chronic cough, a complete laboratory workup including sinus or chest imaging, pulmonary function test, bronchial provocation and induced sputum cytology should be performed in sequence or simultaneously to identify the common causes of chronic cough such as cough variant asthma, upper airway cough syndrome and eosinophilic bronchitis[1,8]. Possible GERC is considered when the patients have the concomitant typical reflux-related symptoms, the other common causes of chronic cough are excluded and the treatment specific to current etiologies fails to resolve cough completely[30]. If the laboratory findings reveal the abnormal reflux, the favorable response to the subsequent standard medical anti-reflux treatment will confirm the diagnosis of GERC. Otherwise, refractory GERC has to be assumed (Figure 1).

The difficulty in diagnosing refractory GERC is to confirm the cause-effect association between reflux and cough. The resistance to medical anti-reflux therapy may be refractory GERC or the ongoing cough is not related to any continuing reflux[4]. Of note, the false refractoriness to PPIs treatment due to poor compliance to treatment should be excluded. It is found that 40% of the patients with gastroesophageal reflux disease are not compliant to PPIs therapy in a large population based study[31]. Except for omeprazole sodium bicarbonate and dexlansoprazole, the traditional delayed release PPIs should be administered 30-60 min before meals to assure the maximal gastric acid inhibition[32]. When the factors are addressed, the possibility of refractory GERC may be increased.

As shown in Table 1, MII-pH is currently a major laboratory examination for the diagnosis of refractory GERC while barium radiographs and upper gastrointestinal endoscopy are not recommended or used only when MII-pH is unavailable because their overall sensitivity is extremely low[32]. With the impedance probes located at different sites of the esophagus, MII-pH can record the changes in the electric impedance induced by the movement of various types of bolus in the esophagus, recognize gas, liquid and mixed reflux based on the impedance value of bolus, and classify reflux as acid and non-acid according to the pH value of refluxate[19,33]. Theoretically, MII-pH has an ability to detect all types of reflux, identify the characters of refluxate and establish a temporal association between acid or non-acid reflux and cough. MII-pH is superior to the ambulatory 24-h esophageal pH monitoring for the diagnosis of GERC in that it provides additional information of non-acid reflux[30]. Recently, it has been reported that the presence of a pathological acid

Table 1 Diagnostic value of laboratory investigation for chronic cough due to gastroesophageal reflux

Diagnostic tests	Indications	Advantages	Drawbacks
Barium radiographs	Not recommended for diagnosis of GERC unless evaluating for dysphagia	High specificity	Extremely low sensitivity
Upper gastrointestinal endoscopy	Not recommended for diagnosis of GERC. Only useful for the detection of erosive esophagitis but not for non-erosive reflux disease	High specificity	Low sensitivity
Ambulatory 24-h esophageal pH monitoring	Able to detect acid reflux but not non-acid (weakly acidic and alkaline) reflux	Relatively high sensitivity	Modest specificity
Multi-channel intraluminal impedance-pH monitoring	Able to detect both acid and non-acid reflux	High sensitivity	Modest specificity

GERC: Gastroesophageal reflux-induced chronic cough.

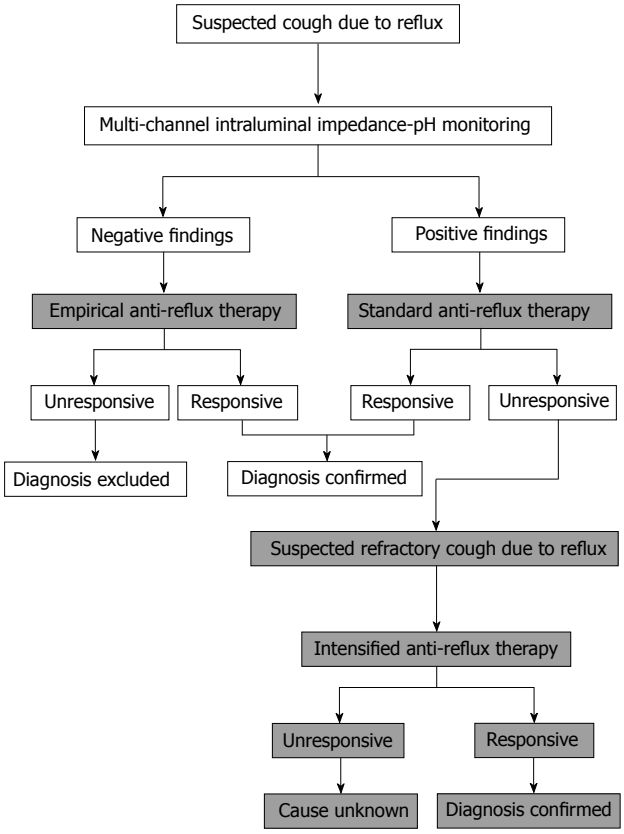

Figure 1 Diagnostic algorithm for refractory chronic cough due to reflux.

exposure time or pathological impedance baseline in MII-pH study may predict the better response to the treatment with PPIs in patients with chronic cough[34]. Therefore, MII-pH has been posited as a future standard for the reflux detection and monitoring[35].

MII-pH has the similar inherent limitations to ambulatory 24-h esophageal pH monitoring. For the estabishment of a temporal association between cough and reflux, the calculation of the symptom association probability (one of significant criteria in the diagnosis of GERC) still depends upon the counts and timing of cough reported by patients and the reflux recorded by MII-pH. Since patients usually underestimate the frequency of cough events or misreport their timing during MII-pH, the symptom association probability determined with above method is not adequately accurate. Several studies have demonstrated that only 40% of cough bursts are indicated by the patients, with a delay of around 30 s, and a positive symptom association probability is found in only 35% of GERC patients[21]. Even though with synchronized intraesophageal manometric monitoring or 24-h ambulatory cough sound recording for precise recognition of cough, the positive rate of the symptom association probability is only improved to 45%-48%[21,36]. Therefore, the sensitivity of MII-pH in the diagnosis of refractory GERC is not high enough to meet the need in clinical practice.

The diagnostic criteria for the refractory GERC can be defined as follows[4,7,8]: (1) chronic cough, with or without the classical reflux-related symptoms such as regurgitation and heartburn; (2) MII-pH confirms the abnormal acid or non-acid reflux, defined as DeMeester score of $\geqslant$ 14.72 and/or the symptom association probability for acid or non-acid reflux of $\geqslant$ 95%. However, our study has shown that $\geqslant$ 80% may be an optimal cut-off value for the symptom association probability and can maintain the better balance between sensitivity and specificity in the diagnosis of GERC[37]; (3) cough fails to improve after 8-wk standard anti-reflux treatment with omeprazole (or equivalent PPIs) at 20 mg twice daily and domperidone at 10 mg thrice daily with life style modification, but responds to the subsequent intensified anti-reflux therapy; and (4) other causes of chronic cough are excluded. When a patient meets all the above criteria, refractory GERC can be definitely diagnosed.

THERAPEUTIC INTERVENTIONS

Refractory GERC can be treated pharmacologically and non-pharmacologically. Currently, the intensified medical anti-reflux treatment is the most common therapeutic option (Table 2).

OPTIMIZATION OF PPIS THERAPY

The modulation of brands and doses of PPIs is a useful strategy for the management of refractory GERC. When a PPI fails, switching to another PPI is possibly effective. Several clinical studies have demonstrated

Table 2 Evaluation of therapeutic options for refractory chronic cough due to gastroesophageal reflux

Therapeutic options	Evaluations
Pharmacologically	
Optimization of PPIs therapy	
Switch to another PPI	Useful for some refractory cough due to acid reflux
Doubling the current dose of PPI	Useful for refractory cough due to severe acid reflux
Add-on therapy	
Histamine H2 receptor antagonists	Useful for refractory cough due to severe acid reflux and night-time reflux
TLESRs inhibitors (baclofen)	Useful for refractory cough due to acid or non-acid reflux resistant to PPI therapy
Gabapentin	Useful for refractory cough due to acid or non-acid reflux resistant to PPI and baclofen therapy
Surgically	
Laparoscopic fundoplication	A treatment option for long-term therapy of refractory cough due to acid or non-acid reflux
Endoscopic therapy or transoral incisionless fundoplication	Not recommended for refractory cough due to reflux on the basis of lack of long-term efficacy
Radiofrequency augmentation	Not recommended for refractory cough due to reflux on the basis of lack of long-term efficacy

PPI: Proton pump inhibitor. TLESRs: transient lower esophageal sphincter relaxations.

that, when adequate symptom relief is not achieved with omeprazole, the switch to esomeprazole (40 mg once daily) for 8 wk may attenuate the symptoms and improve the health-related quality of life in 78% of the patients with gastroesophageal reflux disease[38,39]. This option is also cost-effective. When a single dose of lansoprazole (30 mg once daily) fails, switching to either omeprazole or esomeprazole (40 mg once daily) may achieve adequate symptom control in the patients with gastroesophageal reflux disease[40,41]. However, there is no study to demonstrate the efficacy of a switch to another PPI in patients with refractory GERC.

To increase the dose of PPIs may help to achieve complete acid suppression, and eliminate the residual acid reflux in patients with refractory GERC. Doubling the original dose of PPIs is a common selection. When the dose of lansoprazole is increased from 30 mg daily to 60 mg daily, adequate symptom control is achieved in approximately 20%-30% of patients who are unresponsive to the original low dose lansoprazole for 6-8 wk[40,41]. Our study has demonstrated that the cough in 38.9% of patients with refractory GERC was controlled after the treatment with a doubled-dose omeprazole (40 mg twice daily)[12]. These patients had a more severe esophageal acid exposure, as indicated by a mean 85-point DeMeester score, suggesting the standard dose PPIs were not enough to obviously reduce the acidity of refluxate. After treatment with doubled-dose omeprazole, the significant increase in the pH value of refluxate may markedly attenuate the acid-induced stimulation to the esophageal receptors, inhibit the esophageal-tracheobronchial reflex and finally resolve the cough[12].

ADD-ON THERAPY WITH HISTAMINE H_2 RECEPTOR ANTAGONISTS

Seventy-five percent of patients with refractory gastroesophageal reflux disease still present with abnormal nocturnal gastric acid secretion even after the treatment with PPIs twice daily[42]. The addition of a histamine H_2 receptor antagonist at bedtime may help to achieve complete acid suppression in these patients. Retrospective studies have shown that the combination of PPIs with histamine H_2 receptor antagonists may improve the overall symptoms in 72% of patients with refractory gastroesophageal reflux disease[43]. Since long-term use of histamine H_2 receptor antagonists may develop tachyphlaxis and decrease its therapeutic efficacy, its intermittent or on-demand use at bedtime is advocated. Our results showed the addition of ranitidine 150 mg twice daily attenuated the cough symptoms in 25% of patients with refractory GERC who were unresponsive to the therapy with high dose PPIs[12]. This combined therapy is not to eliminate the night-time reflux but to completely inhibit the day-time gastric secretion by acting on multiple targets in the parietal cells of the stomach. Surprisingly, refractory GERC responsive to the add-on therapy with ranitidine had lower severity of acid reflux (mean DeMeester score of only 36.3) than that responsive to treatment with doubled dose PPIs, suggesting that the esophageal hypersensitivity to acid is a major cause in these patients[12].

ADD-ON THERAPY WITH TLESRS INHIBITORS

Baclofen is a selective gamma-aminobutyric acid (GABA) B receptor agonist primarily used for the treatment of spasticity. It has been demonstrated baclofen can reduce the frequency of TLESRs, decrease the reflux episodes[44-46], and relieve the acid reflux related symptoms by 72% and non-acid reflux related symptoms by 21%[47]. In addition, baclofen has non-specific antitussive activity and has been used for the treatment of refractory chronic cough of unknown causes[48]. As an add-on therapy to PPIs, baclofen may significantly improve the cough symptoms and decrease the cough sensitivity to inhaled capsaicin in 56.3% of patients with refractory GERC[7]. Therefore, baclofen may be useful for treatment of refractory GERC unresponsive to other anti-reflux therapies.

However, baclofen can decrease but not completely abolish TLESRs. It has been documented that baclofen only reduces the frequency of TLESRs by 40%-60% and decrease the reflux episodes by 43%[44-46]. Therefore, the residual reflux may continue to produce cough through stimulating the receptors in the distal esophageal mucosa. This explains why baclofen is not always effective to relieve the refractory GERC. To develop more potent TLESRs inhibitors with few side effects may be a future direction. Furthermore, some refluxes may be secondary to the decreased pressure difference between stomach and esophagus because of the lower baseline pressure of lower esophageal sphincter, and thus be unrelated to TLESRs. Therefore, baclofen is not effective for these refluxes.

The main side effects of baclofen are related to the central nervous system since it can permeate the blood-brain barrier. This limits its clinical application. Nevertheless, the drug-related somnolence, drowsiness and fatigue are usually tolerable and may disappear within 3 wk in most patients. Only a few patients have to stop the baclofen treatment due to severe dizziness and drowsiness[7]. A gradual increase in the dose of baclofen from 5 mg to 20 mg per time may help to improve the patients' tolerance and avoid the severe adverse effects.

ADD-ON THERAPY WITH GABAPENTIN

Gabapentin is a lipophilic structural analogue of GABA, an important central neurotransmitter, and may prevent the synaptic release of neurotransmitters by binding selectively to the $Ca_{v}\alpha_{2}\beta$ subunit of the voltage gated calcium channels. It is primarily used to treat chronic neuropathic pain. Since patients with chronic cough have a similar central sensitization to those with chronic neuropathic pain, the possible inhibition of hypersensitized cough center with gabapentin may be a new therapy for the refractory chronic cough. Ryan *et al*[49] have demonstrated that gabapentin can improve the cough symptoms and cough-specific quality of life in the patients with refractory chronic cough with 8-wk treatment with gabapentin starting at 300 mg daily and titrating up to 1800 mg daily. Madanick *et al*[50] have reported that, after the add-on therapy with gabapentin, approximately 75% of GERC patients experienced at least 50% subjective improvement in cough, irrespective of findings from the esophageal pH monitoring. The dose of gabapentin they used was 300 mg daily in most patients and 900 mg daily or more in a few patients, obviously lower than that reported by Ryan *et al*[49]. At present, it remains unclear whether the cough attenuation after gabapentin therapy is associated with the inhibition of reflux. Further studies are needed to clarify this issue.

ANTI-REFLUX SURGERY

Anti-reflux surgery can treat GERC by artificially reestablishing the mechanical barrier between esophagus and stomach to block both acid and non-acid reflux. Currently, the most commonly used anti-reflux surgery is laparoscopic fundoplication, which can improve more in cough symptom and PPI elimination[51]. In contrast, the radiofrequency augmentation, silicone injection and endoscopic suturing of the lower esophageal sphincter as well as transoral incisionless fundoplication are not recommended due to the absence of evidence supporting their long-term efficacy[32]. The reported successful rate of laparoscopic fundoplication for refractory GERC was about 85%[52]. However, the efficacy of anti-reflux surgery reduces over time. The rate of cough resolution decreases post-operationally from 83% at 6 mo to 74% at 2 years, and 71% within 5 years[53]. Because of its invasiveness and uncertain efficacy, anti-reflux surgery is not a first-line treatment and not extensively used in clinical practice.

In conclusion, refractory GERC is a disorder difficult to manage. Its underlying mechanisms may be associated with incomplete acid suppression, non-acid reflux, TLESRs and esophageal hypersensitivity. MII-pH is a major laboratory examination and can establish the temporal association between reflux and cough, which, however, need to be confirmed by the subsequent intensified anti-reflux therapy. Refractory GERC can be treated pharmacologically and non-pharmacologically. The optimization of PPIs and add-on therapy with histamine H_2 receptor antagonists, TLESRs inhibitors baclofen and gabapentin are the selective pharmacological therapies for refractory GERC. However, the further study is needed to satisfy its management.

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Early probiotics to prevent childhood metabolic syndrome: A systematic review

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author Prof. Sanjay Patole at olof.skoldenberg@ki.se.

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Abstract

AIM: To conduct a systematic review of studies on early probiotic supplementation to prevent childhood metabolic syndrome (MS).

METHODS: Using the Cochrane systematic review strategy we searched PubMed, EMBASE, CENTRAL, CINAHL, and the conference proceedings of the Pediatric American Society meetings and trial registries in December 2014. Randomised controlled trials (RCTs) and non RCTs of probiotic supplementation to the mother and/or infant for a minimum duration of 4 wk were selected. Of these, studies that reported on MS or its components (obesity, raised blood pressure, hyperglycemia, dyslipidemia) in children between 2-19 years were to be eligible for inclusion in the review. Risk of bias (ROB) in selected RCTs and quality assessment of non-RCT studies were to be assessed by the Cochrane ROB assessment table and New Castle Ottawa scale.

RESULTS: There were no studies on early probiotic administration for prevention of childhood MS (CMS). Follow up studies of two placebo controlled RCTs (*n* = 233) reported on the effects of early probiotics on one or more components of MS in children aged 2-19 years. Meta-analysis of those two studies could not be performed due to differences in the patient population, type of outcomes studied and the timing of their assessment. Assessment of childhood metabolic outcomes was not the primary objective of these studies. The first study that assessed the effects of prenatal and postnatal supplementation of *Lactobacillus rhamnosus GG* on body mass index till 10 years, did not report a significant benefit. In the second study, *Lactobacillus paracasei* 19 was supplemented to healthy term infants from 4-13 mo. No significant effect on body mass index, body composition or metabolic markers was detected.

CONCLUSION: Current evidence on early probiotic

administration to prevent CMS is inadequate. Gaps in knowledge need to be addressed before large RCTs can be planned.

Key words: Metabolic syndrome; Obesity; Probiotic; Infant; Perinatal; Children

Core tip: Metabolic syndrome (MS) is a state of dysregulated glucose and lipid metabolism. The global health burden due to increasing prevalence of MS in children and adolescents, warrants urgent preventive interventions. Altered gut microbiota has been implicated in the pathogenesis of MS. The role of maternal and/or infant probiotic supplementation in improving metabolic health of the offspring is being researched. We provide a systematic review of this evidence. Gaps in the knowledge and issues regarding selection of patient population, probiotic intervention and outcomes for future trials have been discussed.

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INTRODUCTION

Metabolic syndrome (MS) is defined to include an array of risk factors that increase the chances of Cardiovascular morbidity and type 2 diabetes in an individual. The World Health Organisation (WHO) in 1998, defined MS to comprise of insulin resistance in the presence of any two of the risk factors - obesity, hypertension, high triglyceride level, reduced high-density lipoprotein cholesterol level, or micro albuminuria[1]. Since then, various definitions for MS have been proposed. MS is increasingly being recognised in children. The diagnostic criteria for MS have been adapted for children and adolescents by the WHO, National Cholesterol Education Program, International Diabetes Federation and I Guidelines for prevention of atherosclerosis in childhood and adolescence[1-4]. There are more than ten different clinical definitions for childhood MS (CMS)[5]. Additionally, there are no unifying criteria that are representative of ethnically diverse groups. The age of onset of CMS is also unclear. A systematic review of 36 epidemiological studies analysing the prevalence of MS in children aged 2-19 years, reported a prevalence ranging from 1.2%-22.6% with rates up to 60% in overweight and obese children[6]. A recent systematic review has reported a mean overall prevalence of CMS as 3.3% (range: 0%-19.2%)[7]. The prevalence was higher in overweight [11.9% (2.8%-29.3%)] and obese [29.2% (10%-66%)] children. It was also higher among males and older children. Higher prevalence has been reported in the Middle East and the United States compared to Europe and the Far East. The variations in the definition of MS, ethnicity, age and nutritional status of the study population may explain the wide range of prevalence reported in these studies. To our knowledge, there is no data available on the health burden of CMS. Follow up of the Framingham Heart study cohort has revealed that the combination of central obesity, hypertension and hyperglycemia led to 2.36 times increase in the incidence of cardiovascular events and three-fold increase in mortality among adults[8]. MS results in a seven fold increase in the risk of type 2 diabetes[9].

Obesity is considered the most important component of CMS. Data from the National Centre of health Statistics, United States reveal that prevalence of obesity has doubled in children and quadrupled in adolescents in the past 30 years[10]. Increased prevalence of CMS is a direct result of the increasing trends of childhood obesity. Cost of illness studies from the United States, Australia, Germany have confirmed that health care utilisation by children with obesity, is significantly higher than their normal weight counterparts[11]. The annual medical expenditure due to childhood obesity in the United States is approximately 14 billion United States dollar (USD) and the projected costs for the next 30 years due to currently prevailing trends of adolescent obesity would be 45 billion USD[12,13]. Thus CMS has the potential to be a major public health concern to both the developed and developing countries[14].

Pathogenesis of MS

Pathogenesis of MS is complex and involves insulin resistance, lipid partitioning, hepatic steatosis, free radical injury and hormonal changes (leptin, adiponectin, resistin)[15-17].

Role of gut microbiota

Numerous reviews have indicated the role of altered gut microbiota in the pathogenesis of MS[18-20]. Gut microbiota (*e.g., bacteroides*) can mediate energy harvest from diet resulting in obesity and type 2 diabetes[21]. Increased level of inflammatory markers (Lipopolysaccharides, Transforming growth factor-β) by gram negative bacteria in the gut can increase gut permeability and oxidant injury and thereby affect the metabolic health[22]. The firmicutes: bacteroides ratio in the gut flora was significantly reduced in children with type 1 diabetes as compared to healthy children[23]. Obesity and excessive weight gain during pregnancy was associated with aberrations in the maternal gut microbiota[24]. Collado *et al*[25] have reported lower stool bifidobacterial counts and reduced microbial diversity in infants born to obese mothers. Follow up of that cohort revealed an increased risk of obesity at seven years of age[26]. It was suggested that early infancy gut microbial alteration could influence metabolic health of children

and adolescents. Gut microbiota is also more amenable to modulation, prior to the establishment of adult type microbiota; *i.e.,* in the first two years of life[27]. Hence, it could be hypothesised that interventions modulating gut microbiota in early infancy can potentially reduce the risk of CMS.

Role of probiotics in prevention of MS

Probiotics are "live micro-organisms which when administered in adequate amounts confer a specific health benefit on the host". Probiotics have been shown to decrease body weight gain, adipose tissue mass, leptin and cholesterol levels. Diet induced hyperglycemia and hyperinsulinemia was controlled by probiotic supplementation. However, majority of the clinical evidence is from adult and animal studies[28-30]. The potential of probiotics in improving metabolic outcomes in children has been studied by maternal and/or early infant supplementation. The pathways for the potential benefits are direct modulation of the infant gut flora through breast milk or placenta and regulation of risk factors such as maternal hyperglycemia and obesity[31-34].

Given the significance of the health issue and the potential of probiotics as an intervention, we aimed to conduct a systematic review of studies reporting on probiotic supplementation to prevent CMS.

MATERIALS AND METHODS

Study selection criteria

The study selection criteria is described as follows: (1) Studies: Randomized controlled trials (RCTs) and non-RCT studies; (2) Participants: Pregnant women and/or infants that received probiotic supplementation for at least 4 wk; (3) Intervention*:* Probiotic supplement of any strain, dose and form with or without prebiotic oligosaccharide for a duration of at least 4 wk; (4) Control*:* Standard treatment but no probiotics or placebo; and (5*)* Outcome measures*:* We broadly defined our outcome measures to account for variations in age, gender and ethnicity based cut offs for individual risk factors of CMS. To be included in this review, the studies should have assessed at least one of the following four components of MS - obesity, raised blood pressure, dyslipidemia (hypertriglyceridemia or low HDL cholesterol), hyperglycemia in children between 2-19 years. The outcome equivalents for each of the components are described as follows: (1) Obesity: body mass index, waist circumference; (2) Hyperglycemia: Fasting plasma glucose, insulin levels, insulin resistance (assessed by homeostatic model assessment for insulin resistance), insulin sensitivity, fasting plasma glucose; (3) Dyslipidemia: Plasma lipid profile (total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, total cholesterol/HDL); and (4) Hypertension: Systolic blood pressure and /or diastolic blood pressure.

We followed the standard Cochrane methodology and the preferred reporting items for systematic reviews and meta-analysis, for conducting and reporting RCTs in this systematic review[35]. We followed the Meta-analysis of observational studies in epidemiology guidelines for conducting and reporting outcomes of non-RCTs in this systematic review[36].

Literature search

We searched the Cochrane central register of controlled trials (CENTRAL) (http://www.cochrane.org/cochrane/hbook.htm), MEDLINE *via* PubMed (http://www.ncbi.nlm.nih.gov/Pubmed), EMBASE (http://www.embase.com) and annual conference proceedings of the Pediatric Academic Societies (www.pas-meeting.org)/ in December 2014. PubMed was searched using two search strategies: (1) *S*tudies of probiotics related to the components of MS using the Medical Subject Heading keywords "insulin resistance" or " insulin sensitivity" or "hyperglycemia" or "type 2 diabetes mellitus" or "obesity" or " overweight" or "adipose tissue" or "dyslipidemia" or " body composition" or "bodyweights and measures" or "hypertension" or "blood pressure" and "probiotics"; and (2) Studies of probiotic supplementation in pregnant women and infants using the MeSH keywords "infant" or "infant, newborn" or "pregnancy" and "probiotics".

For both searches, the MeSH word "probiotics" was replaced by lactobacillus, bifidobacterium and saccharomyces and citations were retrieved. We combined both the search strategies to retrieve studies of probiotics in pregnant women and infants that assessed one or more components of MS as defined for this review. The results of the database search are shown in a flow diagram (Figure 1).

No restrictions were applied on study design or language. Animal studies and studies involving patients > 19 years were excluded. References of the obtained studies were also reviewed to identify additional studies. The international trial registry (www.clinicaltrials.gov) and Australian Clinical Trials Registry (www.anzctr.org.au)were checked for ongoing/registered trials in this area.

Data collection and analysis

Selection of studies: Balasubramanian H and Patole S independently assessed for inclusion all the potential studies identified as a result of the search strategy. Any disagreements about study selection were resolved by discussion.

Data extraction and management: Both the authors independently completed a pre specified data extraction form for all included studies. Any disagreements in the extracted data were resolved through discussion.

Assessment of risk of bias in included studies: Risk of bias (ROB) in selected RCTs and quality assessment of non-RCT studies were assessed by the Cochrane ROB assessment table and the New Castle Ottawa scale[37,38]. Both the authors separately assessed each

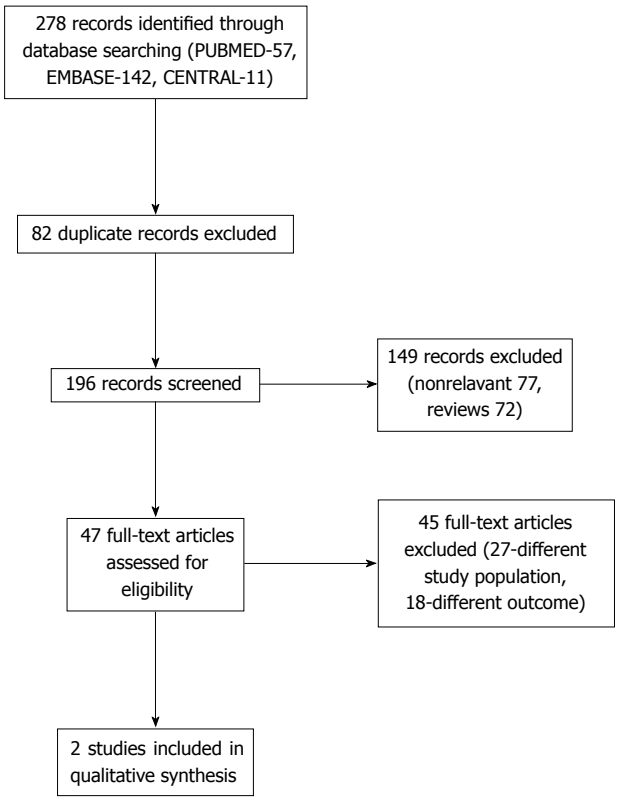

Figure 1 Flow diagram of study selection process.

study. Additional information from the trial authors was requested to clarify methodology as necessary. Any disagreement was resolved by discussion.

RESULTS

Initial broad search yielded 278 citations. We could not find any study on the effects of probiotic administration on CMS. However, we retrieved two RCTs (n = 233) reporting on the effects of early probiotics on one or more components of MS in children aged 2-19 years[39,40] (Figure 1). Meta-analysis of these 2 studies could not be performed due to differences in the patient population, type of outcomes and the timing of their assessment. Hence we decided to conduct a narrative synthesis.

Luoto 2010

This was a follow up study of a double blinded RCT involving 159 mothers with family history of atopic eczema, allergic rhinitis or asthma. They were randomized to receive probiotics (n = 77) or placebo (n = 82). The intervention group received 1 x 10^{10} cfu/d of *Lactobacillus rhamnosus GG* for 4 wk before expected delivery and extending for 6 mo postnatally to the mother/infant.

Frequency of atopic eczema in their children till 2 years of age was the primary end point of the study. The BMI and frequency of overweight and obesity was assessed in 113 children (Probiotic: 59, Placebo: 54) at 2, 4, 7, and 10 years of age. Obesity and overweight was assessed in both groups using the international obesity task force criteria. There was no significant difference in the adjusted mean BMI at any age between the 2 groups. Among the children that were overweight at 10 years, (Probiotic: 13, Placebo: 12), there was tendency towards lower mean BMI at 4 years in the probiotic group (P = 0.063, Analysis of variance for repeated measures).

Videhult 2014

This was the follow up study of a RCT involving 179 vaginally delivered term infants with birth weight > 2500 g. These infants were fed cereals with or without probiotic (*Lactobacillus paracasei ssp F19* - 1 x 10^8 CFU) between 4-13 mo. The outcomes of interest were the number of days with infections and antibiotic prescriptions before and after the second and third doses (5.5 and 12 mo) of DTaP vaccine. A total of 120/179 children were assessed at 8-9 years for the following outcomes-BMI Z score, sagittal abdominal diameter, body composition (fat free mass, fat mass index, truncal fat %, android or gynoid fat %), plasma lipids, insulin, glucose and transaminases. No significant differences in body composition, growth and metabolic markers were noted in the two groups at 8-9 years of age.

Results of the ROB assessment are reported in Table 1.

DISCUSSION

Our systematic review identified two RCTs (n = 233) studying the effects of early probiotic supplementation on metabolic health in children. Meta-analysis of these 2 studies could not be performed due to differences in the patient population, type of outcomes studied and the timing of their assessment. The current evidence on the administration of probiotics to the mother or infant to prevent CMS is thus inadequate.

To our knowledge this is the first systematic review assessing the role of early probiotic supplementation in the prevention of CMS. Small number and sample size of the included studies was the main limitation of this systematic review. Included studies were not designed to study metabolic outcomes in children and had follow up losses of up to 30%. Considering the global burden of CMS and the metabolic benefits of probiotics in adults and animal models it is important to assess this intervention in large RCTs. Few issues need to be discussed with regards to the patient population, probiotic intervention and outcome assessment in such trials.

Selection of the infant population for such trials is crucial as the current evidence on benefits of probiotics with regards to CMS related outcomes is based on healthy term infants. Preterm infants and those with intrauterine growth restriction are at high risk for MS due to catch up growth and reduction in insulin sensi-

Table 1 Assessment of risk of bias in the included studies

Study ID	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Other bias
Luoto *et al*[40]	Low	Low	Low	Low	High	Low	Low
Videhult *et al*[39]	Low	Low	Low	Low	High	Low	Low

tivity[41,42]. Infants of diabetic mothers are also at higher risk of MS[43]. Factors that put these infants at high risk of early infancy gut microbial aberrations include increased risk of caesarean delivery, prolonged hospital stay, decreased maternal contact, perinatal and/or postnatal antibiotic exposure, delayed enteral feeding, need for tube feeds, formula feeding and suboptimal nutrition[44]. Hence research should focus on these high risk infant groups, and maternal population, especially obese and diabetic mothers. Comprehensive assessment of gut flora and immunological profile would also be essential as they relate to the mechanisms/pathways of benefit of probiotic supplementation. Considering that the effects of probiotics are strain specific and host specific, selection of probiotics is an important issue. A comparative meta-analysis by Million *et al*[45] has shown that Lactobacillus acidophilus administration resulted in significant weight gain in humans and in animals and Lactobacillus gasseri was associated with weight loss both in obese humans and in animals. The same authors have also reported that obesity-associated gut microbiota is rich in Lactobacillus reuteri and depleted in Bifidobacterium animalis and Methanobrevibacter smithii[46]. Assessment of the effects of probiotics on body composition is helpful considering the nutritional benefits of probiotics[47,48]. Assessment of optimal timing and duration of intervention are also important issues in the RCTs of early probiotic supplementation for preventing CMS. Rinne *et al*[49] have demonstrated that probiotic administration during the last 6 mo of pregnancy and first 6 mo postpartum did not influence long term (2 years) composition of the infant gut flora. Perinatal metabolic programming and immune mediated effects on the infant gut flora by the administration of probiotic could explain the pathway of benefit[50]. Controlling for confounders (*e.g.*, dietary and lifestyle changes), assuring compliance during the prolonged period of supplementation, and monitoring for complications will be necessary[51-53]. Currently there are no universally accepted criteria for defining CMS or its components[54]. Selection of primary outcomes representative of some or all components of MS would be essential. Since the minimum age cut off described for CMS is unclear, standardisation of surrogate end points will be essential. Currently there are no studies showing a causal relation between MS and gut microbiota. Moreover, the risk factors for MS differ with age. Prematurity, low and high birth weight, rapid catch up growth, maternal undernutrition, maternal obesity and diabetes are potential risk factors for the components of CMS. This highlights the necessity to test early interventions (perinatal, early postnatal) for preventing CMS.

In summary, current evidence is insufficient to assess the effects of probiotics in reducing the risk of MS in children and adolescents. Considering the global health burden of CMS and the potential role of a low cost intervention such as probiotic supplementation, clinical and epidemiological studies are urgently required in this field. Better understanding of the pathogenesis and population specific cut offs of the various components of CMS is required before high quality randomised trials can be undertaken to address this important issue.

COMMENTS

Background

Metabolic syndrome (MS) in children and adolescents is defined to include central obesity, hyperglycemia, high blood pressure and dyslipidemia. Probiotics have shown to reduce adipose tissue, glucose and triglyceride levels in animal models, but evidence in children and adolescents is insufficient.

Research frontiers

Observational studies have shown that altered gut flora in infancy is associated with obesity in childhood. Altered gut flora has also been noted in children with type 1 diabetes. Whether modulation of gut flora in early infancy by probiotic supplementation would decrease the risk of childhood obesity and glucose intolerance is unclear.

Innovations and breakthroughs

Evidence from a clinical trial suggests that perinatal probiotic interventions may decrease the risk of gestational diabetes and central obesity. A prospective study of perinatal probiotic supplementation in early infancy has shown to reduce the risk of excessive weight gain in obese children. However, data on childhood metabolic outcomes is limited. Currently there is insufficient evidence to support the role of early probiotics in childhood MS (CMS).

Applications

To date, there is no systematic review on early interventions to prevent CMS. Given the magnitude of the problem, they analysed the potential role of probiotic exposure in early life for prevention of CMS or its components. The cause - effect relationship of altered gut flora *vs* CMS needs to be studied. High quality RCTs analysing all components of CMS are required.

Peer-review

Definitely this is an interesting topic and is properly argued by the authors. Unfortunately they could not present a meta-analysis because of the data available in the literature. However, it is important to publish this work to emphasize the urgent need for this kind of research.

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SYSTEMATIC REVIEWS

Prevalence of antibiotic resistance in *Helicobacter pylori*: A recent literature review

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Abstract

AIM: To review previous studies (the last 6 years) about the *Helicobacter pylori* (*H. pylori*) antibiotic resistance in order to evaluate the trend in antibiotic resistance.

METHODS: In this study, the PubMed, MEDLINE, Science Direct, Google Scholar and Scielo manuscripts were reviewed from 2009 to 2014.

RESULTS: On the whole rates of *H. pylori* antibiotic resistance were 47.22% (30.5%-75.02%) for metronidazole, 19.74% (5.46%-30.8%) for clarithromycin, 18.94% (14.19%-25.28%) for levofloxacin, and 14.67% (2%-40.87%) for amoxicillin, 11.70% (0%-50%) for tetracycline, 11.5% (0%-23%) for furazolidon and 6.75% (1%-12.45%) for rifabutin. The frequency of tetracycline, metronidazole and amoxicillin resistance was higher in Africa, while clarithromycin and levofloxacin resistance was higher in North America and Asian, respectively.

CONCLUSION: The most sensitive drug is rifabutin and the lowest sensitive drug is metronidazole in the world. The worldwide *H. pylori* antibiotic resistance to clarithromycin and levofloxacin has increased during the last 6 years. The present systematic review show alarming results and a novel plan is needed for eradication therapy of *H. pylori* infections.

Key words: Antibiotic resistance; *Helicobacter pylori*; Worldwide

Core tip: Because of the rising frequency of antimicrobial resistance, management of *Helicobacter pylori* (*H. pylori*) infections is a challenge for physicians. We found global frequency rate of resistance is high in Africa. The

most sensitive drug is rifabutin and the lowest sensitive drug is metronidazole in the world. The worldwide *H. pylori* antibiotic resistance to clarithromycin and levofloxacin has increased during the last 6 years. Resistances to antimicrobial agent's reports describe dramatic decrease of antibiotics efficacy.

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INTRODUCTION

Helicobacter pylori (H. pylori) is a motile, curved and Gram negative bacillus[1]. *H. pylori* certainly is the most prevalent human infection, the frequency of infection due to *H. pylori* is nearly 50% in the world and in developing country is as high as 80%-90%[2]. This bacterium colonizes the stomach of human and its infection is correlated with gastritis, peptic ulcer disease and extra-digestive diseases[3,4]. *H. pylori* is also considered as a human carcinogen[5]. Since, *H. pylori* eradication therapy represents a key clinical essential. Unfortunately, therapy against *H. pylori* has turned out to be more difficult over the years, principally due to the great decrease of standard eradication therapies efficacy.

Although *H. pylori* is sensitive to many antibiotics *in vitro*, just a few antibiotics can be used *in vivo* to treat infected patients. Management of *H. pylori* infections are recommended in all suggestive individuals[6]. According to the latest Maastricht Guidelines, in regions of low clarithromycin resistance, clarithromycin-containing treatments are recommended for first-line empirical treatment[7]. In regions of high resistance to clarithromycin, the quadruple treatment including bismuth has been proposed as first-line treatment. In case of unavailability of this therapy, non-bismuth (three antibiotics plus Proton pump inhibitors) quadruple therapy and the so-called "sequential therapy" (that includes five days of PPIs plus amoxicillin followed by five more days of PPIs plus metronidazole and clarithromycin) have been recommended as an alternative[7]. Table 1 is shown mode of actions and resistance mechanisms of antibiotics used for treatment of *H. pylori* infection.

Failure of treatment in *H. pylori* infections has become an actual subject for physicians. The cause of treatment failure is many that can be grouped into microorganism-related factors, host-related factors and treatment-related factors. *H. pylori* resistance to antibiotic is widely recognized as the chief reason for treatment failure[1,8]. Furthermore, antibiotic resistance should be considered as a lively idea, since its prevalence can change not only among diverse countries, but also between two different periods in the same area[1,9-11]. The rate of antibiotic resistance in *H. pylori* has been evaluated worldwide. However, most researches originated from single center, included only a small number of bacteria, were often restricted to selected patients, and used different techniques to evaluate antibiotic susceptibility. Though, the investigation platform is luxurious; and only performed in few countries as: United Kingdom, German, Finland[12-18]. Antibiotic use for infections other than *H. pylori* is accounting for the extensive raise antibiotic resistance rate in *H. pylori*[19]. Because of the value of *H. pylori* therapy, antimicrobial susceptibility testing has been widely done. Since, *H. pylori* antibiotic resistance is fast growing worldwide, an eradication policy based on pre-treatment susceptibility testing is going to get more attractive than in the past[1,7].

The objective of this paper was to review previous studies about the rates of antimicrobial resistance in *H. pylori* isolates obtained from worldwide during last 6 years in order to evaluate the trend of antibiotic resistance.

MATERIALS AND METHODS

In the present study, different computer-assisted searches were achieved using PubMed, MEDLINE, Science Direct, Google Scholar and Scielo. Separately searches were carried out on all English language literatures published through 2009 to 2014, by the key words: *Helicobacter pylori*, *H. pylori,* resistance, metronidazole, levofloxacin, amoxicillin, clarithromycin, tetracycline, and rifabutin. Full articles related searches were saved, and articles written in foreign languages were translated when essential. When more than one publication from the same author was obtainable, only new version, counting the whole population was enrolled. Two investigators (Ebrahimzadeh Leylabadlo H and Mohammadzadeh Asl Y) independently and in a blinded manner assessed the articles using pre-designed data extraction.

The following information was collected: (1) sum of bacteria incorporated; (2) rate of antibiotic resistant; and (3) the geographic area involved. The data were summarized in extraction table and analyzed manually. Finally, Excel 2007 software was used to draw charts.

RESULTS

During 6 years a total of 52008 *H. pylori* isolates meeting the inclusion criteria were identified. Eighty-seven studies from 2009 to 2014 on *H. pylori* antimicrobial resistance in the different countries were included; there were 43 Asian[20-62], 10 American[63-72], 5 African[73-77], and 29 European studies[78-106]. On the whole rates of *H. pylori* antibiotic resistance were 47.22% (30.5%-75.02%) for metronidazole, 19.74% (5.46%-30.8%) for clarithromycin, 18.94% (14.19%-25.28%) for levofloxacin, and 14.67% (2%-40.87%) for amoxicillin, 11.70% (0%-50%) for tetracycline, 11.5% (0%-23%) for

Table 1 Mode of action, resistance mechanisms of antimicrobial agents used for treatment of *Helicobacter pylori* infection

Antibiotic	Mode of action	Resistance mechanisms
Metronidazole	Electron reduction processes, leads to the formation of nitro-anion radicals and subsequent DNA damage	(1) Poor drug uptake and/or increased drug efflux; (2) enhanced activity of DNA repair enzymes; (3) increased oxygen scavenging abilities; and (4) decreased antibiotic activation arising from changes in metronidazole-reducing enzymes[16]
Clarithromycin	The inhibition of protein synthesis by binding and slowing down the activity of the bacterial ribosomal unit[17]	rRNA-point mutations
Amoxicillin	The inhibition cell wall synthesis	*pbp* gene mutations, membrane permeability alterations and efflux pumps[17]
Tetracycline	Reversible inhibition protein synthesis	Three contiguous nucleotides mutation in the 16S rRNA gene[17]
Fluoroquinolones	Inhibiting DNA gyrase, type Ⅱ topoisomerase, and topoisomerase Ⅳ[17]	Point mutations in the quinolones resistance determining regions
Rifabutin	Inhibits the b-subunit of *H. pylori* DNA-dependent RNA polymerase encoded by the *rpo*B gene[18]	Mutation of the *rpo*B gene[18]

H. pylori: *Helicobacter pylori*.

Table 2 Antibiotic resistance rates in different continental areas

Region (*n*)	Cla %	Amo %	Met %	Tet %	Lev %	Rif %	Fur %
Asia (23748)	27.46	23.61	46.57	7.38	25.28	12.45	23
South America (587)	12.88	6.56	52.85	0	21.23	NR	0
North America (818)	30.8	2	30.5	0	19	NR	NR
Europe (26024)	22.11	0.35	31.19	1.15	14.19	1	NR
Africa (831)	5.46	40.87	75.02	50	15	NR	NR
Total (52008)	19.74	14.67	47.22	11.70	18.94	6.75	11.5

Amo: Amoxicillin; Cla: Clarithromycin; Met: Metronidazole; Tet: Tetracycline; Lev: Levofloxacin; Rif: Rifabutin; Fur: Furazolidon; *n*: Number; NR: Not reported.

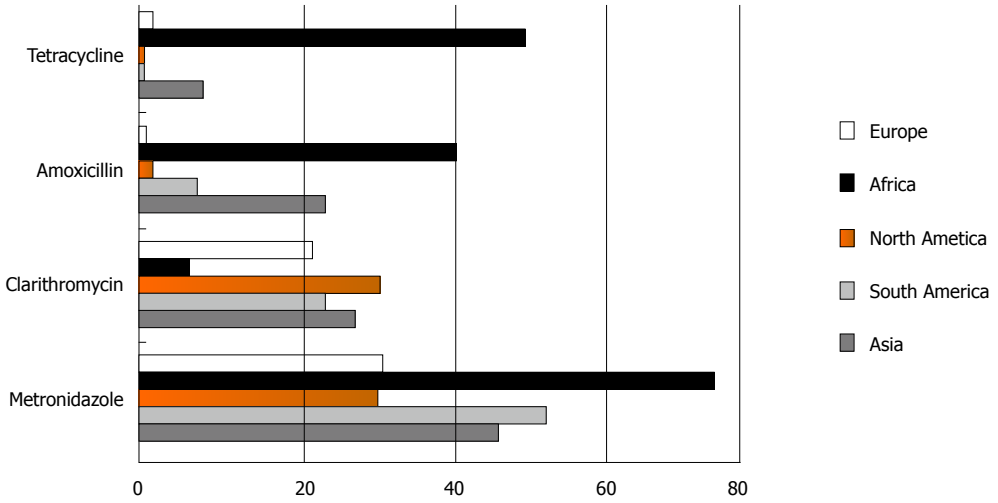

Figure 1 Antibiotic resistance rates to 4 most common used antibiotics in different continental areas.

furazolidon and 6.75% (1%-12.45%) for rifabutin. The frequency of resistance to antibiotics in various continents and countries are demonstrated in Tables 2 and 3, Figures 1 and 2.

DISCUSSION

Monitoring of resistance to antimicrobial agents is important for *H. pylori* infections therapy in medical practice[17]. Resistance to antimicrobial agents creates at risk *H. pylori* eradication in the world[10,98]. The most recent recommendations on *H. pylori* therapy suggested that initially management had better be personalized based on clarithromycin and metronidazole resistance. In fact, fourteen days triple-therapy is recommended in area where resistance to clarithromycin is more than 15% to 20%, if resistance to metronidazole is more than 40%, the association with amoxicillin is

Table 3 Quantitative data of the articles

Countries	Year	Isolates (*N*)	Cla (%)	Amo (%)	Met (%)	Tet (%)	Lev (%)	Rif (%)	Fur (%)	Method	Ref.
	2014	95	33.7							E-Test	[20]
	2013	82	17.1	9.8	64.4	0				DDM	[21]
	2013	78	15.3	6.4	55.1					DDM	[22]
Iran	2012	150	34	10	78.6	9.3	5.3			E-T,ADM	[23]
	2012	112	14.3	28.6	76.8	18.7		28.6		DDM	[24]
	2011	197	45.2	23.9	65.5	37.1			61.4	DDM	[25]
	2011	42	14.3	2.4	40.5	4.8				ADM	[26]
	2010	121	5	20	44	3				E-Test	[27]
	2010	132	30	6.8	73.4	9				E-Test	[28]
	2014	73	80.8	0	58.9		12.3			E-Test	[29]
	2013	17731	21.5	0.1	95.4		20.6		0.1	ADM	[30]
China	2011	73	84.9	0	61.6	0	13.7	6.8		PCR	[31]
	2010	374	37.2	0.3	63.9	1.2	50.3			E-Test	[32]
	2009	36	8.3	33.3	94.4		0		16.7	DDM	[33]
	2014	124	36.2	0	2.1					E-Test	[34]
Japan	2014	135	25.9		20.7					E-Test	[35]
	2014	1073	31.1		40.2					ADM	[36]
	2013	204	86.4	8.2	71.3		57			ADM	[37]
	2011	153	55.6							PCR	[38]
	2010	61	36.1	0	14.8					ADM	[39]
	2014	212	8.5	9	36.3	0				ADM	[40]
South Korea	2013	165	11.5	2.45	50.7	0	24.55			ADM	[41]
	2013	150		6						ADM	[42]
	2012	185	10.8	2.2	30.3	0.05				ADM	[43]
	2014	161	1.2		36.6					E-Test	[44]
Malaysia	2014	102	6.8	0	32.3	0	6.8	0		E-Test	[45]
	2011	90	0	0	75.5		0	14.4		E-Test	[46]
	2011	187	2.1	0	37.4	0	1			E-Test	[47]
	2009	187	2.1							E-Test	[48]
	2014	46	47.8	54.3	73.9	4.3				E-Test	[49]
Pakistan	2012	178	36	37	89	12				ADM	[50]
	2010	92	33	2	48					E-Test	[51]
	2014	98	23.5	3.9	11.7					DDM	[52]
	2012	149	18.2	0	45.5		18.2			E-Test	[53]
Turkey	2012	61	21.3	0	42.6	9.1	3.3			DDM	[54]
	2009	31	41.9	3.2	41.9	3.2				E-Test	[55]
	2009	38	13.5							ADM	[56]
Taiwan	2014	61	35.3	0	17.6	0	23.5			E-Test	[57]
	2009	180	10.6	0	26.7		9.4			E-Test	[58]
Thailand	2009	120	29.2							PCR	[59]
UAE	2010	26	19.2							E-Test	[60]
India	2014	80	58.8	72.5	83.8	53.8	13.8		13.8	DDM	[61]
Vietnam	2013	103	33	0	69.9	5.8	18.4			E-Test	[62]
South American											
	2014	54	11.1	1.9						E-Test	[63]
Brazil	2013	77	19.5	10.4	40	0			0	ADM	[64]
	2011	39	8	0	51	0	23		0	ADM	[65]
Colombia	2012	203	19.8	20.5						ADM	[66]
Cuba	2010	40	10		85					E-Test PCR	[67]
Peru	2011	95					36.9			ADM DDM	[68]
Uruguay	2009	79	8.9	0	35.4	0	3.8			E-Test	[69]
North America											
Mexico	2011	90	5.5		19					E-Test	[70]
Canada	2009	42	57							E-Test	[71]
United States	2011	686	30	2	42	0	19			E-Test ADM	[72]
Senegal	2013	108	1	0	85	0	15			E-test DDM	[73]
Nigeria	2009	186		66	95	100				E-test	[74]
Gambia	2012	64	0		68.8					ADM	[75]
Tunisia	2010	273	15.4	0	51.3					E-test	[76]
South Africa	2010	200		97.5						ADM DDM	[77]
	2014	1651	6.7		29.4					E-test	[78]

Germany	2013	5296	67.1	0	67.1		24.9		E-test	[79]
	2013	436	7.5	0	32.7		11.7		E-test	[80]
Italy	2012	111	35.2		59.3		22.1		E-test	[81]
	2011	253	9.9						PCR	[82]
England	2009	255						1	E-test DDM	[83]
	2013	343	23.5		33				E-test	[84]
Spain	2011	71	14.7	1.4	45.1	0	14.5		E-test	[85]
	2010	118	35.6						E-test	[86]
	2009	101	54.6		35.7				E-test	[87]
Norway	2012	102	5.9	0	22.5	0			E-test	[88]
Finland	2010	505	8	0	41		7		E-test	[89]
	2013	588	20.1		34.5	2.6			ADM	[90]
Bulgaria	2011	519	17.9		29.5	4			ADM	[91]
	2009	1057	18.7	0.5	21.35	3.15			ADM	[92]
Croatia	2012	382	11.9	0.6	10.1				E-test	[93]
	2014	210					8.1		E-test	[94]
Poland	2013	165	10.9		32.7		1.2		E-test	[95]
	2012	51	22				16		E-test	[96]
	2011	115	34	0	44		5		E-test	[97]
Portugal	2014	180	50	0.6	34.4	0.6	33.9		E-test	[98]
	2011	1115	34.7	0	13.9	0			E-test	[99]
Belgium	2013	189	13.3	0.8	26.1					[100]
	2011	10670	20.3	0	27				ADM	[101]
Netherlands	2014	417	6.14		10.1				E-test	[102]
	2013	746	20.5	0.68	19.9				E-test	[103]
Ireland	2013	85				0	11.7	0	E-test	[104]
	2010	219	13.2		31.5				E-test	[105]
Southern Europe	2014	74	34.7		16.7				E-test	[106]

Amo: Amoxicillin; Cla: Clarithromycin; Met: Metronidazole; Tet: Tetracycline; Lev: Levofloxacin; Rif: Rifabutin; Fur: Furazolidon; DDM: Disk Diffusion Agar; ADM: Agar Dilution Agar.

preferred[17]. At the present, due to *H. pylori* antibiotics resistance, eradication therapy appears was not carried out as simple as and we are now founded many failures which make the use of standard therapy unacceptable in many parts of the world[107]. This article systematically studied the latest data on *H. pylori* resistance to antibiotic.

Clarithromycin resistance

Because clarithromycin is the most potent antibiotic involved in the management of *H. pylori* infections, resistance to clarithromycin is important[8,17,105]. As presented in Table 2, the rate of clarithromycin resistance was 19.74%, and occurrence of clarithromycin resistance is increasing worldwide (Figure 2). The rate of clarithromycin resistance has been broadly studied, and information are on hand from nearly all areas in the world: it ranges from 5.46% to 30.8% (Figure 1).

In European regions, the lowest clarithromycin resistance was reported from Norway (5.9%), whilst the highest in Spain (32.01%) and Portugal (42.35%). European studies performed at the past 6 years intervals reported that *H. pylori* resistance decrease from 36.65% in 2009 to 24.38% in 2014. In Asian regions, a surprising clarithromycin resistance frequency was reported from India (58.8%) and China (46.54%), whereas the lowest rate was discovered in Malaysia (2.4%). An increase in clarithromycin resistance has been faced in the Asia, from 15.28% in 2009 to 32.46% in 2014, probably in the Asian countries macrolid drugs used more. In recent years due to widespread use of clarithromycin for respiratory infections in the public especially in children, clarithromycin resistance has augmented in diverse regions, and there is an association between outpatient use of long-acting macrolide and clarithromycin resistance[10,17,108].

In conclusion, the highest clarithromycin resistant area was North America, and this study showed a slight increasing tendency of clarithromycin resistance of *H. pylori* in the world. Since clarithromycin is the most potent antimicrobial agent involved in the standard treatment protocol as well as the resistance rates were still at the low level, where clarithromycin-containing triple therapies could be used empirically.

Metronidazole resistance

Metronidazole is used against *H. pylori* infections and is one of the few antibacterial agents as drug of choice that is effective in eradicated the microorganism. Some researcher reported that the rate of treatment failure is more than 20% with triple therapy in which metronidazole is the drug of choice, also *H. pylori* resistance to metronidazole is the chief solitary reason responsible for management failure[109,110].

Metronidazole resistance is the most common antibiotic resistance in *H. pylori* and overall metronidazole resistance found in 47.22% in descending order in Africa 75.02%, South America 52.85%, Asia 46.57%, Europe 31.19%, to 30.5% in North America. In developed countries about 30% of the *H. pylori* strains

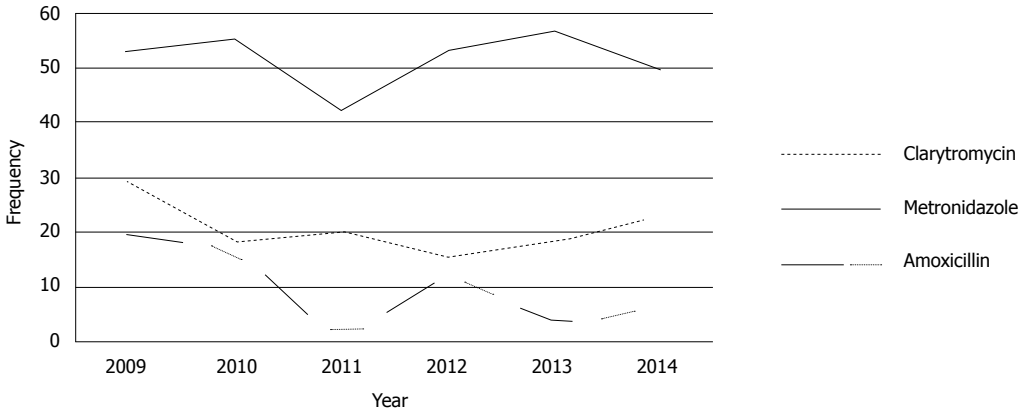

Figure 2 Trend of *Helicobacter pylori* resistance to metronidazole, clarithromycin, and amoxicillin during 6-years.

are metronidazole resistant, whereas in developing countries, the occurrence of resistance is very high. This association between metronidazole resistance and socioeconomic state level is maybe due to use of metronidazole and related drugs for gynecological, dental and parasitic related infectious diseases[13,111]. The comparison of results indicated that resistance to metronidazole have remained significantly unchanging in Asian, European and North American countries but is increasing in African countries (51.3% in 2010 to 85% in 2013). Furthermore metronidazole resistance in 2014 has stayed approximately at the similar level as in early 2009 in Europe. So, in accordance with latest guidelines, metronidazole is favored to amoxicillin in first-line therapy in Asian, Europe and North American but not in African patients.

Amoxicillin resistance

Amoxicillin is suggested for anti-*H. pylori* triple therapy in region where metronidazole resistance is high. Universal resistance to amoxicillin is uncommon; it was detected in 14.67%. The frequency of amoxicillin resistance extensively differs in Asian regions, ranging from zero in Malaysia, Taiwan and Vietnam to 72.5% in India. The rate of amoxicillin resistance in Africa was 40.87%.

The prevalence of amoxicillin resistance in Europe countries and North American is low from zero in certain area as Finland, Germany, Norway and Poland, 1.4% in Spain to 2% in United States. It seems the government policy possibly to limit the use of antibiotic for infectious diseases in European and North American countries. The incidence of amoxicillin resistance in *H. pylori* seems to increase specially in Asia and South America, where these antibiotics can be obtained without prescription. *H. pylori* resistance rates of 97.5%, 72.5%, 66% and 20.5% for amoxicillin have recently been reported in South Africa, India, Nigeria and Colombia, respectively.

Tetracycline resistance

Among the 4 most common used antimicrobial agents, tetracycline resistance was the lowest (Table 3). In general *H. pylori* resistance to tetracycline was detected 11.7% in the world. The total rate of tetracycline resistance did not vary in South America and North America (the resistance was absent), whilst it was relatively high in Africa (50%). In Asia, the resistance was absent in Thailand, and very low in China (0.6%) and South Korea (0.01%). In contrast, increased values were found in India (53.8%), and Iran (11.7%). The prevalence of tetracycline resistance stays very low (less than 7.4%) in almost most parts of the world except for Africa. The comparison of data showed that tetracycline resistance is decreasing in the world, 26.85% in 2009 to 6.11% in 2014.

Tetracycline is a bacteriostatic and broad spectrum antimicrobial agent that is active against *H. pylori* and tetracycline is the most generally used antibiotic for treatment of *H. pylori* and other infectious diseases[109]. Tetracycline is extensively used in many countries, but resistance to this antibiotic has not become a great problem yet. Management failure owing to the tetracycline resistant has been reported[112,113], though there is not enough data obtainable until now to determine the impact of this resistance on management success.

Rifabutin resistance

However, the study on *H. pylori* rifabutin resistance is inadequate and in South America, North America and Africa has not been done during previous 6 years. The rate of rifabutin resistance was higher in Asia (12.45%) as compared to Europe (1%). The frequency of rifabutin resistance differs in Asian countries, ranging from 28.6% in Iran to about 7% in China and Malaysia. Rifabutin is structurally related to rifampin group, and it has potential efficacy against *H. pylori*[114]. Rifabutin is usually used to treat mycobacterium diseases, so the secondary resistance of *H. pylori* to rifabutin is not currently expected in the healthy people.

Levofloxacin resistance

Generally, resistance to levofloxacin is low (< 19%) worldwide. The prevalence rate was higher in Asia

(25.28%) and South America (21.23%) as compared to Africa and Europe (less than 15%). The frequency of levofloxacin resistance widely differs in Asian regions, about 57% in Japan, 24.55% in South Korea, 5.3% in Iran and 2.6% in Malaysia. In addition the levofloxacin resistance rate differs between European countries, ranging from 7% to 33.9%. The rate of levofloxacin resistance seems to be increasing universal from 4.25% in 2009 to 17.55% in 2014. Furthermore, during the past 3 years levofloxacin resistance rates have even been more increasing.

Due to the dramatic increase in clarithromycin resistance, levofloxacin, a wide spectrum quinolone, has been used as an option of clarithromycin in some regimens. But the frequent use of quinolones for urinary tract infections has increased the incidence of *H. pylori* resistance in the world[17]. Failure of therapy due to levofloxacin resistance and the emerging development of quinolones resistance, use of levofloxacin as first-line therapy is generally discouraged, and its utilize should be reserved as a second-line or save regimens after failure of a clarithromycin and/or a metronidazole based regimen[7,80].

Furazolidon resistance

The study on furazolidon resistance was not widely performed in the world, and in Europe, North America and Africa has not been achieved during past 6 years. The rate of furazolidon resistance was higher in Asia (13.8%) as compared to South America (0%). The rate of furazolidon resistance broadly differs in Asia, from 61.4% in Iran to 16.8% in China and 13.8% in India. Furazolidon is a cheap and synthetic nitrofuran with a wide spectrum activities usually used in the treatment of bacterial and protozoa infections. Since high *H. pylori* resistance to metronidazole in some region as China and South America, furazolidon sometimes has been used as an option for *H. pylori* infections[65]. However some researchers were reported that the rate of cure with furazolidon-based regimens is low and a large amount of furazolidon increases the therapy rate but it significantly raises complications[81].

The prevalence of *H. pylori* metronidazole resistance is at a high level, and resistance to clarithromycin and levofloxacin is increasing worldwide. The most effective drug is rifabutin and the lowest sensitive drug is metronidazole. Resistance to levofloxacin does not show any region difference. There are no studies regarding rifabutin and furazolidon resistance of *H. pylori* in America and Africa. According to the present findings, the mean resistance rate in *H. pylori* isolated from European and North American patients is lower than other countries. The rate of tetracycline, metronidazole and amoxicillin resistance is higher in African patients, while clarithromycin and levofloxacin resistance is higher in North America and Asian patients. In conclusion, antibiotic resistance is increasing, so empirical therapy must be based on information of antimicrobial drug resistance, and this paper highlight a steady worldwide surveillance of *H. pylori* antibiotic resistance.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) is a most important human pathogen associated with significant disease and fatality.

Research frontiers

Due to the rising frequency of antimicrobial resistance, management of *H. pylori* remains a challenge for physicians in most parts of the world.

Innovations and breakthroughs

Search was carried out about *H. pylori* antimicrobial resistance literatures published through 2009 to 2014.

Applications

The frequency of antibiotic resistance is increasing, and this article highlight a steady worldwide surveillance of *H. pylori* antibiotic resistance.

Peer-review

This is a systematic review article on *H. pylori* resistance to antibiotics. The manuscript is well written and the topic of interest.

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EDITORIAL

Conflicts of interest in nutritional sciences: The forgotten bias in meta-analysis

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Conflict-of-interest statement: (1) Dr. Lucas is Assistant Professor at Université Laval since July 2012, and Visiting Scientist at the Harvard T.H. Chan School of Public Health. Full salary of Dr. Lucas comes from Université Laval and CHU de Québec, and is cover by a salary award from the Fonds de recherche du Québec - Santé (FRQS); (2) Between 1999 and 2012, while he was a student, Dr. Lucas often spoke at conferences (mainly on omega-3 fatty acids). His honoraria and expenses were covered by private industries. He has never received research funding from private industries; (3) Dr. Lucas has no relationships with entities that might have an interest in the submitted work; (4) Dr. Lucas' spouse, children and partners have no financial relationships with the submitted work; and (5) Dr. Lucas does not have any non-financial interests in the submitted work.

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Abstract

Awareness of conflicts of interest (COI) in medicine began in the 1980s. More recently, the problem has gained notoriety in nutritional sciences. COI with industry could bias study conclusions in the context of research activities and scientific publications on nutritional sciences. The issue of COI in nutritional sciences deserves more attention and requires careful analyses as biased information can negatively impact the development of dietary guidelines and, ultimately, population health. Decision-making is generally based on available, published evidence, but when the results are ambivalent, it is easier to opt for the status quo and ask for more studies. Readers might wonder if research is subsidized by industry as a counterbalancing strategy based on levels of evidence-only to slow down eminent positions and/or legislation on the food sector? How can this problem be overcome without producing paranoia and McCarthyism while trying to be as methodological as possible?

Key words: Conflicts of interest; Nutrition; Nutritional sciences; Bias; Systematic reviews; Meta-analysis

Core tip: Decision-making in the field of nutrition is based on published evidence, but when results are ambivalent, it is easier to opt for the status quo and ask for more studies. Because conflicts of interest (COI) in nutritional sciences can bias conclusions and negatively impact dietary recommendations and population health,

it deserves more attention and requires careful analyses. To regard evidence properly and in a rigorous manner, COI in systematic reviews and meta-analyses must be evaluated systematically to guarantee the trustworthiness of nutrition-related studies, and must therefore be obligatory sub-analyses to reduce the risk of bias in data interpretation.

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INTRODUCTION

Thompson[1] defined conflicts of interest (COI) as "a set of conditions in which professional judgment concerning a primary interest (such as a patient's welfare or the validity of research) tends to be unduly influenced by a secondary interest (such as financial gain)". To researchers, financial incentive is not necessarily the only influent interest. The desire for recognition, academic advancement, success in publication and funding are other powerful stimulants[2]. Scientists delight in believing they are immune to and very mindful of COI. According to Young[3]: "We are not always aware of our own biases. The idea that scientists are objective seekers of truth is a pleasing fiction, but counterproductive in so far as it can lessen vigilance against bias." The purpose of this editorial is to discuss justifiable concern about the COI problem in nutritional sciences and the need to seriously take it into account with critical reading of scientific journals and inclusion of its analysis in systematic reviews (SR) as well as meta-analyses (MA). The nature of COI and human behavior relevant to COI are beyond the scope of this editorial, as they have already been well-described by other authors[3,4].

Even if we cannot accurately ascertain the beginnings of COI investigation in medicine, its consideration intensified in the 1980's[5,6] and it still continues[7]. In 2009, the Institute of Medicine dedicated a full report to COI, indicating that its concerns are justifiable[8]. Corruption of healthcare by Big Pharma is a long-standing debate, but the one on Big Food is much more recent[9]. Indeed, *PloS Med* and *BMJ* recently published a complete series on the food and beverage industries, their influence and COI[10,11]. To Loder[10], the industry-researcher relationship "is not evidence of research malpractice. It does, however, contribute to perceptions that nutrition science might be for sale." However, nutritional sciences are not the exclusive domain of COI. Such biases are well-known in the tobacco[12] and pharmaceutical industries[2], and parallels also apply to the food industry[13].

Although most scientific journals instruct authors to report all COI, not all published studies declare them. Lesser *et al*[14] noted that 54% of scientific articles - relating to drinks (beverages, juice, and milk) and published between 1999 and 2003 - named their financial sponsorships. They assessed the influence of funding bias by determining the relationship between industry (sponsorship) funding (yes, no, mixed support) and the conclusions of scientific articles (favorable, unfavorable or neutral). They reported an odds ratio of 7.61 (95%CI: 1.27-45.73) for favorable *vs* unfavorable conclusions in all industry-funded articles compared to those without industry funding. They concluded that "industry funding of nutrition-related scientific articles may bias conclusions in favor of sponsors products, with potentially significant implications for public health". Diels *et al*[15] scrutinized the relationship between COI and study outcomes (favorable/unfavorable) in the realm of genetically-modified food products, using similar methodology. They found that financial COI were not associated with the results, but discerned strong linkage between professional COI (author affiliation with industry) and study outcomes. Bes-Rastrollo *et al*[16] examined relationships between COI and food companies, conclusions on sugar-sweetened beverage (SSB) consumption, and weight gain in published SR. Among the 6 SR that identified "COI with food companies", 83.3% (n = 5) reported no positive linkage between SSB intake and weight gain, whereas among the 12 SR that found "no COI with food companies", 83.3% ascertained positive associations. They noted that studies with "COI with food companies" were 5 times (relative risk = 5.0; 95%CI: 1.3-19.3) more likely to present no positive association between SSB consumption and weight gain than those without COI. These contradictory findings do not, however, establish which SR is right, but they clearly indicate discrepancies, depending on whether or not COI exist.

Drug studies have advantages over those in nutrition-the results are first submitted to government agencies for scrutiny before drug approval. Therefore, data from unpublished drug research are available, but this is not the case in nutrition. Moreover, by accessing trials registered with the United States Food and Drug Administration[17], the European Medicines Agency[18] and other government bodies, it can be determined if they have been published or not, if more negative studies are unpublished, if published results agree or conflict with agency decisions, and if there is risk of publication bias. Although not perfect, different methods detect and correct for publication bias[19,20]. However, unless bias is severe, these tests have low power and high false-positive rates in perceiving significant asymmetry[19]. Therefore, no statistical methods are superior to any others in assessing publication bias and they should be viewed as exploratory analyses. Publication bias in SR and MA is related not only to published and unpublished studies, but also to factors which influence published studies (*e.g.*, statistical significance, study size and quality, type of study design, *etc.*)[21]. COI are often forgotten factors.

To help in the critical analysis of published SR and MA,

guidelines such as PRISMA[22] (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) should be followed. The publication of protocols for SR and MA in the Cochrane Library (http://community.cochrane.org/) or Prospero (http://www.crd.york.ac.uk/prospero/)[23] could ensure rigorous methodology. Although these tools are driven by methodological markers, they do not constitute an analysis of quality in themselves. Peer review committees and scientific panels do not always guarantee the quality of published studies. If included studies are of poor methodological quality, their results are likely to be biased. Therefore, the outcomes of SR and MA could be linked with high risk of bias, even if the methodology is implemented with great rigor. The Cochrane Statistical Methods Group and the Cochrane Bias Methods Group developed a risk of bias tool that focuses on 6 domains: Selection, performance, detection, attrition, reporting and other biases[24]. However, this risk of bias tool seems insufficient to capture biases related to study funding sources[25]. A Cochrane Review found that the risk of bias between drug industry and non-drug industry-funded studies was similar, but observed that drug industry-funded studies reported more "favorable" results than non-drug industry-funded studies[26].

As mentioned earlier, the problem of COI is complicated by the fact that not all studies sufficiently declare sponsorships and the financial affiliations of authors. Indeed, in the 2010 Cochrane Database of Systematic Reviews, a very low proportion of published drug studies reported funding sources (30%, 46/151 reviews), author-industry financial ties or employment (11%, 16/151 reviews)[27]. These findings are overwhelming, considering that Methodological Expectations of Cochrane Intervention Reviews Item No. C44 (Describing Studies) is mandatory[28]. Funding sources and COI declarations by primary researchers should be collected in this process and appear in the table on "Characteristics of included studies".

As suggested by Bero[25]: "The impact of the bias can be assessed descriptively or by using subgroup analysis, comparing industry-funded to non-industry-funded studies, as is commonly done in Cochrane Reviews. A bias should not be ignored even if we do not fully understand its mechanism, just as we should not ignore harms of interventions if we don't understand how they arose, or ignore the harm of smoking because we don't know how smoking causes cancer. Therefore, a study's funding source should be evaluated as an independent risk of bias." As pointed out by Rothman[29], there is a risk of McCarthyism with labeling of scientists as having COI. He recommended "that a work should be judged solely on its merits" and "We can halt this new McCarthyism in science and get back to focusing on the work of a scientist rather than on his or her life story".

To regard evidence properly and in a rigorous manner, COI in SR and MA must be evaluated systematically to guarantee the trustworthiness of nutrition-related studies, and must therefore be obligatory sub-analyses to reduce the risk of bias in data interpretation. COI may influence the results not only by showing statistically significant associations between exposure and disease, but also by demonstrating lack of associations, especially among groups that protect interests by inducing doubts and claiming unproven causation. Surprisingly and unfortunately, none of the MA in nutritional sciences assessed COI - to the best of the author's knowledge while writing this editorial! Therefore, SR and MA must include sub-analyses that try to examine if studies with COI: (1) industry-sponsored; (2) authors-industry-affiliated; and (3) sponsorship or author affiliation - have more favorable outcomes (results, conclusions) than other investigations. The methodology employed by Diels *et al*[15] for SR and by Lundh *et al*[26] for MA could establish definitions, such as: (1) Sponsorships: industry-funded, non-industry-funded, and unknown/unclear sponsorship; (2) Authors' affiliations: Industry-affiliated, non-industry-affiliated, and unknown/unclear; and (3) Classifications that combine sponsorship and author affiliations: COI (sponsorship or author affiliations), no COI, and unknown/unclear.

Nutrition is one of the most vital health determinants of society, not only in regard to the etiology of chronic diseases, but also because it is an important target for public health interventions. Investment in epidemiological approaches - allowing rigorous study into the roles of both individual and overall diets in disease risk - is undoubtedly a key to success. However, to continue to attract interest and trustworthiness, nutritional sciences must be faultless.

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EDITORIAL

Toward phase 4 trials in heart failure: A social and corporate responsibility of the medical profession

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Abstract

Congestive heart failure (CHF) is a chronic condition, requiring polypharmacy, allied health supports and regular monitoring. All these factors are needed to ensure compliance and to deliver the positive outcomes demonstrated from randomized controlled trials. Unfortunately many centers around the world are unable to match trial level support. The outcomes for many communities are thus unclear. Research design factors in post-marketing surveillance to address this issue. Phase 4 studies is the name given to trials designed to obtain such community level data and thus address issues of external validity. CHF phase 4 studies are relatively underutilized. We feel the onus for this research lies with the health profession. In this commentary we provide arguments as to why phase 4 studies should be viewed as a social and corporate responsibility of health professional that care for clients with CHF.

Key words: Clinical trial; Corporate responsibility; Health system; Congestive heart failure; Phase 4

Core tip: This commentary brings needed and timely attention to phase 4 or postmarketing surveillance. Only a handful of congestive heart failure (CHF) therapies have actually been studied in the community after the randomized controlled trial. In this millennium it is important we not only innovate and support trials of new therapies, but also ensure the therapies we are already using are effective for all patients. As drug discovery and randomised controlled trial evidence is often done by private sector pharmaceuticals, we thus feel the need to bring attention on treating health care teams to regularly generate efficacy and effectiveness data for the CHF treatments they prescribe.

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INTRODUCTION

"…'medical Profession' consists of the sum of the identities of its membership … if the 'profession' is held responsible for something, each of its members is responsible, in some way, for it". -French PA

It is the collective responsibility of the medical profession to seek and administer processes to account for past practices and improve future health practices. The practice of medicine, today, is a complex process that has to factor many considerations. An important consideration is the evidence based practice. This evolution initially saw prescribing that was based on uncontrolled observations of physiology in individuals, to controlled observations on groups. The pursuit of this new goal has become quite complex and corporatized, such that we often forget some of the basics that has safely steered the profession. All systems also have to factor the social and ethical contracts between governments, and its citizens, demanding equitable health services, or risk community wrath at the ballot box. The prescription of pharmaceuticals is one arm of this complex process. Many pharmaceutical manufacturers operate from the private sector. A financial investment is made in developing a drug, where there is always the risk that it may not provide the necessary benefits, hence unmarketable. To standardize this competition with accountability, the randomised controlled trial (RCT) has been used to generate the evidence base. Should there be positive findings, the company that has made that investment now has the legitimacy to market the product. There are however limitations with this process. In this editorial we discuss the importance of continuing governance once the drug is approved for community use. In this, all health professionals have to ask the hard questions and truly understand the entirety of their responsibilities not only in the delivery but the governance of clinical as well as the corporate issues. Let us look at examples for this from several vantage points that are timeless.

ETHICS

Whenever a doctor cannot do good, he must be kept from doing harm. -Hippocrates

Safety, the pillar of medical practice, ingrained at the start of training and reminded at graduation with the "Hippocratic Oath", is a social contract we as health care workers (HCW) have with our clients. Before a HCW does anything we should firstly do no harm. As soon as a HCW starts their duties whether it is consulting, diagnosing, prescribing, dispensing, delivering, promoting or preventing, they run the risk of doing harm. There is no way to determine risk-benefits ratios of any intervention without adequate checks and balances. Medication errors are the eighth leading cause of death in the United States. Cardiovascular medications account for a large proportion of these errors, predominately as inpatients in the emergency department and acute hospital settings. Errors include omissions, incorrect dosage, under prescribing, and failure to consider adverse interactions. Errors are more likely to occur when clinical workload is heavy; there is language, communication, cultural barriers; although, generally the majority of these errors occur from a lack of intention[1]. Geographical distances and unavailability of services are not factored much in guidelines to achieve simplicity of the therapeutic regime. Universal and standardized reporting of errors and adverse side effects has been in play from many health bodies and centralised to government bodies to ensure accountability. Standardization of knowledge and training at undergraduate and continuity thereafter are also important measures to reduce this risk[2,3]. System wide the monitoring of this, however, remains inadequate.

STANDARDS

"…The art of medicine was to be properly learned only from its practice and its exercise…" -Thomas Sydenham

Health regulators have ensured that delivery of health care has a minimum basic standard through law and enforcement by regulatory bodies. Such examples include the medical universities, training colleges, therapeutic goods admirations, medical councils and overseas trained doctor regulators. At the core of this is the curriculum. As health information and technologies are evolving so must HCW and systems. Continuous medical education (CME) is now required by medical bodies to maintain up-to-date knowledge, although barriers remain. Among a small number of general practitioners, barriers were identified in many dimension of care[4]. With system wide barriers it will be difficult for regulators to introduce standards for improvement at the level of the health clusters. In addition there are silos between the administrators, HCW and clients. This often makes it difficult for HCW to practice in-sync with advancements, while using their local experience. This in fact is a translational block that occurs far too often. In-fact these local experiences in the practice of medicine are not given any emphasis. No doubt HCW may use this in their practice, perhaps unregulated, without the knowledge of how it is translating. As an example, Joynt *et al*[5] highlighted the differences in mortality outcomes between physicians managing a high *vs* low volume of congestive heart failure (CHF) cases. These benefits were noted regardless of age, sex, race and comorbidities. Such care was also more intense, and with greater use of skilled nurse and rehabilitation. However, readmission rates were higher[5]. Identifying priorities is one reason. The experience of HCW also appears vital for improved outcomes. In the real world

there remain many clusters that never achieve this high volume status, and where admitting patients more frequently are not possible. How we learn from these positive examples, how we disseminate that knowledge and how we use technology to share workloads to achieve the adequate standards and outcomes are issues health systems must address. To truly factor in experiences, guidelines need to achieve consensus and standardize sections in it that reflects on the benefits of regional variations in practice. Creating options of how such variations can be created, while ensuring all these deviations are audited is an important standard to set in shaping CHF guidelines of the future. Another issue of concern when dealing with fixed guidelines is its generalizability.

GENERALIZABILITY

"We should be concerned not only about the health of individual patients, but also the health of our entire society". -Benjamin Carson

It is important we pay attention to the generalizability of standards and guidelines. Rothwell, highlighted two vital questions when evidence is gathered through RCTs: firstly, are the results valid for patients other than from the trials; and are the results generalizable to similar patients but in a different treatment setting[6]. Due to the way trials are set up, it will be impossible to test every conceivable permutation (scenario) while controlling biases. Cultural sensitivities, an example of one such, are important areas to negotiate. Often HCW exercise judgement which on occasion could flirt the boundaries of such guidelines or the skills they are thought[7]. This art of medicine touches greatly on subjectivity and relies on the HCW intuition or perhaps experience. In a study of general practitioners if was found that prescribing closely of HF guidelines varied inversely with age[7]. While the younger group could relate to familiarity with guidelines, the importance of age and experience, perhaps related more to on the ground realities in different communities, and may have influenced these differences. There are unfortunately no universal ways to standardize this, but we can still account for this. As highlighted earlier experience can be a factor that affects outcomes. From this HCW may start being creative in their administration of health services. It is not only important that there be accountability but also sharing of this experience to reflect in the published literature.

EXPERIENCE

"It is much more important to know what sort of a patient has a disease than what sort of a disease a patient has". -William Osler

No two health care workers will be identical in the way they practice medicine. Experiences of both client and physicians are important as they tailor their individual views on health and illness. The undisputed common outcome is perhaps the ability to "…live a long, productive and quality life…". Again studies have shown that the higher the volume of HF clients seen by physicians, outcomes tended to be better, suggesting the importance of clinical experience[5]. While we allow doctors to practice in this fashion, we find it difficult to find guidelines that describe medical care in this fashion. Similarly client experiences are important. The clients view on how they are treated in the medical system will reinforce their attitudes to health. These views will have cultural and socioeconomic slants, factors that are not often factored into RCT or guidelines. It may not be as simple in all cases to tell a patient that the medicine you are giving them is the best and they ought to comply regardless of how they feel about it[8,9]. In this sense two powerful qualities are relationships which is vital to develop a good understanding, and choice, which is needed to provide for a holistic HCW-patient experience.

RELATIONSHIPS

"The good physician treats the disease; the great physician treats the patient who has the disease". -William Osler

It is universally accepted that most clients and systems rate HCWs who provide a holistic approach to medical care. There is a spectrum of what is considered holistic including partnerships and seeking of expertise when unavailable. It is also imperative that HCW look out for and form partnerships with other specialists in their own right. Knowledge is not the right of one person and no one group has all the knowledge. To provide a comprehensive client experience all HCW at some point must seek the assistance of their colleague. This area becomes more complex when skills are outside the health cluster. Forming relationships with centres of excellence will help. Technology can be used to bridge such gaps. Translational blocks for these are administrative, requiring a new mind set from all parties. Similarly health services with a greater density of HF specialist were associated with improved outcomes[10,11]. These are other reasons for such partnerships. Good client HCW relationship is an independent marker of positive outcomes[12]. The National institute of health which advocates for improved evidence translation discusses the continuation of "Bench to Bedside" research. In the second arm, "Bedside to Bench", clinical and basic research are equally important in the delivery. It is the obligation of the HCW to seek these out, to improve translation of evidence or to generate greater evidence should it be required. Healthy clinical and scientist HCW relationships are an obligation in pursuit of the optimal client services, where there is also adequate choice.

CHOICE

"Never go to a doctor whose office plants have died". -Erma Bombeck

HCW and clients alike need choices, and the freedom to make informed choices. This includes the

choice of doctor, therapies and the manner in which all aspects of their care are delivered. Guideline based care and the Western paradigm of ambulatory chronic condition care has subverted this process, subtlety such that both HCW and clients deliberate from a small basket of choices[12,13]. We are acknowledged that "a one shoe fits all" approach does not work. Having choices is not an easy process as it requires extension of evidence beyond a Bench to Bedside approach. In the vast majority of cases a guideline based approach is sufficient. In perhaps one in ten cases there is a need for a more creative approach. This may involve using a medication with improved activity for a comorbidity, that is easier to use, that has potentially less side effects. Drug companies that develop the evidence will often stop after the RCT is concluded. In the conclusion of the presented findings it is often written to imply a wide generalizability when in actual fact the results apply to the chosen population treated. It is often left to the HCW to generate this evidence. This involves a competitive process from formulation of the research question to grant funding applications for investigator initiated research. Again this process could be simplified where regulatory authorities directly approve the investigator initiated research and provide the formulary medication. Clearly health economics is a factor and this needs to be discussed. A new approach is also encouraged. Understanding how clients and HCW interact within clusters could be important in reducing this block.

COST

"A hospital bed is a parked taxi with the meter running". -Groucho Marx

"There are no free lunches", "the age of entitlement is over", are choruses that can be heard loudly at political rallies. Escalating health care costs are a concern. The increase in pharmaceuticals prescribed will see funds diverted from other essential services. The easiest way to save cost is to keep the client healthy, prevent future illnesses and reduce tertiary hospital utilization. CHF alone utilizes close to 2% of health care costs, in the United States estimated at close to $35 billion dollars. Among 1054 CHF clients, when this cost is broken down, after a mean follow-up of 4.6 years 73% had died. The estimated lifetime costs were close to $110000 where more than three fourths the costs are accumulated from hospitalizations. The majority of HF patients will suffer at least one other chronic comorbidity[14,15]. As there are overlaps with care within the chronic ambulatory care model ways can be found to minimize this overlap and reduce duplication. This is one simple way. Other options include preventing the treatment of one illness affecting the outcome of another. In these cases we have previously discussed how extra-class effects of drugs, their variable pharmacological properties could be better suited some clients. It is important we generate this evidence at the community level.

MORAL RESPONSIBILITY

"Modern medicine is a negation of health. It isn't organized to serve human health, but only itself, as an institution. It makes more people sick than it heals". -Ivan Illich

Do medical professionals have responsibility beyond the description of their respective professions? There have been in our time physicians who have stood out more than others. Many of these, some included in the above quotes, point to an unwritten, subjective obligation that comes closer to the spiritual than the scientific domain. Regardless of our view points on this we have to accept that the practice of medicine is an inexact science. We are there for obligated to keep an open mind and to continuously strive for improvement in our clients welfare. This can be defined as common sense by pragmatist; of interest by health systems focused on cost and a moral imperative by others. CME is one arm of this where HCW increase their armament for service delivery. Prospective clinical audits are another arm where the focus is on the health system. They should be run by centralised committees within local health clusters controlling the key performance indicators. Six domains from preadmission, emergency, admission, discharge, community and the dimensions of care in each of these domains should be negotiated. An agreed framework for data mining will allow for better and quicker access to information. Health systems in the future should work on standardising the entry, sharing of data and allow for easier access to prospective health data so that local situations can be addressed quicker. We must always find way to provide a frame for the picture to the painted. The barriers here are often administrative and jurisdiction. It is a moral imperative we balance our views on this, prevent silos and look for commonalities. From this picture we can execute our social responsibility as citizens. Thus HCW have responsibilities "…Beyond their call for Duty…".

SOCIAL AND CORPORATE RESPONSIBILITY

"The physician's highest calling, his only calling, is to make sick people healthy-to heal, as it is termed". -Samuel Hahnemann

It is important that systems ask socially responsible questions, *e.g.*, should we provide health services to a community for a decade what is the expected outcome at the end of that time? Is there anything we should do differently? Do we have the adequate skills? Phase 4 studies are a continuation of research once evidence is generated from a RCT. They seek to advance the translational factors such as access, organizational and client factors that could hinder delivery of best practice. Such research could understand both clinical and economic

issues relevant to health clusters and health systems. It provides both information and hypotheses generating questions and should be part of continuous quality improvement[16-18]. It is our collective responsibility to ask these post marketing questions such as: What are the strains on the health systems? What can I or we as a group do to help? When and where to seek assistance? When and where to draw attention? It is thus a social and corporate responsibility for HCW to continue to audit their work to look for better and cheaper ways to provide health services moving into the future.

In summary, to provide medical services and reinforce the RCT findings is a complex process with many factors at play. Health systems have to factor all these. It may seem difficult as such it may be easy to merely do the same. In fact with very simple measures we could achieve an improved standard of medical care. We feel that this standard involves a dedicated emphasis by HCW and systems for post marketing surveillance to address issues within health clusters[19-25]. There are technological advancements now to ensure that this process need not be as laborious as it previously was. Data storage, data mining and standardization of key performance indicators in HF suggest that a subtle shift in thinking and an investment in technology could prove useful. The future must encompass a dedication to regular audits to inform a dynamic CME education curriculum, by breaking down of silos, and embracing technology[26-30]. More powers be given and greater accountability requested from heath clusters by preventing translational blocks. Phase 4 trials should not be viewed as a rigorous process, a vindictive process where some are rewarded and other punished. It should be viewed as a process to generate evidence, improve service delivery, understands subtle local variations, inform the health cluster and add to the global pool of knowledge. Doing so will not only reduce costs but uphold the social contract between providers and recipients of health care.

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REVIEW

Immunodiagnosis of human hydatid disease: Where do we stand?

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Abstract

Cystic echinococcosis (CE) is a zoonotic parasitic infection caused by the larval stage of *Echinococcus granulosus*. Diagnosis of CE mainly relies on a combination of serological testing along with imaging approaches. A variety of serological methods, mainly based on hydatid cyst fluid, antigen B (AgB) and antigen 5, have been developed and used for immunodiagnosis of CE, yet their performances are not satisfactory. Although utilizing of recombinant or synthetic antigens, improved the performance of serological tests, it has not applicably overcome the problem of low sensitivity and cross reactivity, seen in the diagnosis of CE. Performances of immunodiagnostic tests based on AgB subunits are promising. The 8 kDa subunit of AgB is the most studied antigen in native, synthetic or recombinant form for diagnosis of CE. From the 5 subunits of AgB, antigen B8/1 and B8/2 provided the highest diagnostic sensitivity and specificity. Moreover, detecting of specific antibodies of IgG subclasses has improved the efficacy of immunodiagnostic tests. Among the IgG subclasses, both IgG2 and IgG4 are considered as good markers for diagnosis and IgG4 as a suitable marker for follow up of the patients. In this review an overview of immunodiagnostic methods, related antigens and their performances in the diagnosis of CE are given. The paper highlights pitfall and challenges in the serological diagnosis of CE. Moreover, limitation of currently available immunodiagnostic tests and the most recent development in the designing and application of serological assays for diagnosis of CE in human are addressed.

Key words: Immunodiagnosis; Cystic echinococcosis; Hydatid cyst

Core tip: Cystic echinococcosis (CE) (hydatid cyst) is one of the most important parasitic diseases, causing tremendous morbidity and mortality for the human patients. Diagnosis of CE mainly relies on ultrasound images of the cyst along with serological testing. So far, there is no highly specific and sensitive immunodiagnostic test for diagnosis of CE

and performances of the currently available tests are not satisfactory. Different antigenic sources including hydatid cyst fluid, antigen B and 5, excretory-secretory antigens of larval stage or adult worm have widely been used for development of serological assays for diagnosis of CE. Utilizing of antigen B subunits in immunodiagnostic tests and detection of IgG subclasses, as a good marker, opened a promising perspective in diagnosis of this debilitating disease.

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INTRODUCTION

Cystic echinococcosis (CE), known as hydatid cyst or hydatid disease, is a zoonotic parasitic infection caused by the larval stage of *Echinococcus granulosus* (*E. granulosus*). Dogs and other canids harbor the adults tape worm and herbivores acts as intermediate host and become infected through ingestion of parasite's eggs. Human acquire the infection by accidental ingestion of *E. granulosus* eggs.

CE with its significant economic and medical impact constitutes an important public health problem in many developing countries[1-3]. An estimated 1.2 million people worldwide are affected by CE and the disease accounts for annual estimate of 3.6 million DALYs (disability adjusted life years) through the world[4]. Early and proper diagnosis of CE can provide appropriate management and suitable treatment of the disease[5].

Diagnosis of CE is mainly confirmed through a combination of relevant history, serological testing, along with imaging approaches. A variety of serological methods have been developed and used for immunodiagnosis of CE in recent years, including indirect hemagglutination (IHA), immunoblotting, enzyme-linked immunosorbent assay (ELISA), indirect fluorescent-antibody (IFA), latex agglutination test, and immunochromatography test[1,6-11]. For the development of these assays different antigens from adult worm, protoscolices, worm eggs or hydatid cyst fluid have been defined, purified and evaluated in the aforementioned serological tests.

Diagnosis of CE has drastically improved during the last two decades. Progress in methods for antigen purification, cloning expression and purification of *E. granulosus* recombinant antigens, and defining and synthesis of immunodominant peptides contributed to this development. Nevertheless, immunodiagnosis of CE is still problematic. Commercially available serological tests show unsatisfactory performance. The lack of standardization of immunodiagnostic assays and also antigen preparation contribute to discrepancy in results reported in different laboratories. Cyst size, stage and location as well as patients characteristics may be accounted for the discrepancy of the same test performance in different clinical diagnostic laboratories.

Hence, serological assays still have a complementary role to imaging in the diagnosis of CE. Low sensitivity (up to 30% of false negativity) and also low specificity (up to 25% of false positivity) make serological results difficult to interpret[12-17].

Pitfalls and challenges in the diagnosis of CE

In spite of the development of a variety of immunodiagnostic test, following diagnostic pitfalls and challenges still exist in the diagnosis of CE.

Available immunodiagnostic tests give a relatively high rate of false-negativity. False negative results in immunodiagnostic tests for CE may be seen in patients with small cysts, intact cysts, cysts in extrahepatic locations, heavily calcified cysts (*e.g.,* non-viable), or cyst in privilege sites (brain or eye). Akbulut *et al*[18] reported that 15 out of 40 patients with pancreatic echinococcosis, found in the literature have had negative serological testing for CE. Among 65 CE patients in Germany, false negative serological results were reported in 18% by IHA and in 15% by ELISA[19]. In a study by Akcam *et al*[20] more than 20% of patients with extra-hepatic cysts were reported to be negative by IHA test. Using WB, 10 cases of IHA-negative were found to be positive. In a study by Wuestenberg *et al*[21], CE was confirmed in 9 cases of IHA-negative by clinical findings and imaging (US). Cardiac hydatid cyst, with 54 mm × 45 mm size, was serologically negative in Canpolat *et al*[22] report. Karakasli *et al*[23] reported a case of large spinal-para spinal hydatid cyst with negative ELISA and WB testing. They suggested that clinical and neuro-radiological findings should be considered in such cases. Review of 100 case of pulmonary hydatidosis by Zapatero *et al*[24] revealed that positive serological test have been present with ruptured cyst (positive IHA in all of ruptured cyst) while the test detected only 80% of patients with unruptured cysts. Serological test for CE have been negative in human immunodeficiency virus (HIV)-positive cases[25].

Currently available tests give rather high rate of false-positive reaction in patient infected with other parasites (notably cestodes) or even in healthy subjects. False positive results are related to cross reactant antibodies.

Differentiation of past (cured or calcified cyst) from present (active or progressive) hydatid infection is difficult by existing antibody detection assays. Antibody titer may remain for years, even after surgical removal of the cyst or proper drug treatment[26,27]. Therefore a positive serological test may not necessarily imply the presence of active cyst or even the reactivation of CE.

Hydatid cysts in unusual locations may complicate its diagnosis. Congenital, choledochal and pancreatic pseudocysts along with lipoma, ovarian intra-abdominal cystadenoma and intra-hepatic haematoma may be misdiagnosed as hydatid cyst by ultrasonography/computerized tomography (US/CT)[13]. In all of these

conditions an appropriate serological test would be quite helpful with negative results.

Performance of serological tests varies in different pathological stage of CE according to WHO classification[28].

A single defined molecule may not be sufficient for diagnosis of CE. Recent immunoproteome analysis of hydatid cyst fluid (HCF), in different stages of cyst (based on WHO classification), revealed that specific immunodominant epitopes changes from[29] one stage to another stage. This indicates that more than one defined immunodominant antigen may be needed to diagnosis CE in different status of the cyst.

Antigenic sources for immunodiagnosis of CE

Antigenic sources which have widely been used for immunodiagnosis of CE are HCF, component of HCF, ES of protoscolices or adult worm, and also extract of adult worm or larval stage. Antigen for immunodiagnosis of CE has been comprehensively reviewed by Carmena *et al*[12]. The main antigens for diagnosis of CE and their performance in diagnosis of CE are discussed below.

HCF: HCF is the most common antigenic source which has been used for diagnosis of CE. HCF is a mixture of host (albumin, globulins) and parasite components[30-32]. Sensitivities of serological tests based on HCF are high but their specificities are far from satisfactory (30%-90%)[8,10,12,28,31,33-35]. Many of available commercial kits are using HCF in ELISA system for diagnosis of CE. Table 1 summarizes the performance of HCF in diagnosis of CE in different serological assays.

Using HCF as a source of antigen, Sedaghat *et al*[35] evaluated the performance of a simple dot ELISA and CCIEP (counter current immunoelectrophoresis) for diagnosis of human hydatidosis and found a sensitivity and specificity of 100% and 89.1% for Dot-ELISA and 80% and 62% for CCIEP. Dot ELISA had a better performance in comparison with CCIEP. Using HCF, EL-Shazly *et al*[8] reported a sensitivity of 96.7% and specificity of 97.5% for ELISA and 86.7% and 95% for IHA. Al-Sherbiny *et al*[6] applied the camel HCF in a dipstick assay and reported a high diagnostic sensitivity (100%) and specificity (91.4%).

CCIEP is a relatively sensitive, but not specific method for diagnosis of CE. In a retrospective study conducted by Sadjjadi *et al*[15] hospital records of 1227 surgically proven CE cases were examined and found that only 62% of cases had a CCIEP positive test in comparison with 96.3% of positive findings by US and pathology.

Antigen 5: Antigen 5 (Ag5) is one of the most immunogenic and abundant part of HCF. It composed of 57 and 67 kDa components and dissociate into 38 and 22-24 kDa subunits under reducing conditions[36]. Ag5, after AgB, is one of the most studied antigens in the serodiagnosis of CE. Numerous studies pointed out that Ag5 has a high rate of cross-reactivity with sera of healthy controls or other non-CE patients[12,17,37]. Its performances in native, recombinant or synthetic forms have not been satisfactory due to either low sensitivity (50-54), or specificity because of cross reactivity with sera of the patients with other cestoda, trematoda or even nematoda. In an Ag5-based ELISA, Khabiri *et al*[38] reported that IgE and IgG4 are the most important antibodies, with low cross reactivity with sera of healthy control and non-CE cases.

Contrary to these reports, a recent study by Pagnozzi *et al*[39] demonstrated that highly enriched Ag5, by chromatographic method, attained highly specific and unambiguous results, in Western blotting and ELISA system in diagnosis of CE. The authors indicated that low performance of this antigen in previous studies is related to non-properly purified antigen which have been used and considered that highly purified Ag5 is a promising antigen in diagnosis of CE. Having said that, the low number of sera tested in their study does not allow drawing a decisive conclusion. Table 2 shows the performance of Ag5 in diagnosis of CE in different serological assays.

Antigen B: Antigen B (AgB) is a thermostable polymeric lipoprotein of 120-160 kDa, composed of 8 kDa subunits which dissociates into 8/12, 16 and 24 kDa subunits, under reducing condition in SDS-PAGE[36]. AgB is considered as the main antigen of HCF with high specificity and sensitivity in serological diagnosis of CE[9,10,31,40-42].

AgB is highly immunogenic, a feature that makes this antigen a suitable candidate for immunodiagnosis of CE. The smallest subunit, 8 kDa, considered as the most appropriate antigen in diagnosis of CE. Not surprisingly, the 8 kDa subunit of AgB is the most studied antigen in native, synthetic or recombinant form for diagnosis of CE. Sarkari *et al*[42] obtained diagnostic sensitivity and specificity of 100% and 80% when AgB was evaluated in an immunoblotting system. In their study from 40 sera of hydatidosis patients, 32 cases (80%) detected the 8 kDa subunit, 29 cases (72.5%) recognized the 16 kDa component and 29 cases (72.5%) detected the 24 kDa subunit of antigen B. In continuation of their study, when the AgB was used in an ELISA system, sensitivity of the system was determined to be 92.5% and the specificity was found to be 97.3%[10].

Recombinant AgB are not doing much better in diagnosis of CE when compared with native homologues antigens. The performance of rAgB subunits for diagnosis of CE was evaluated by Jiang *et al*[40] where they reported performance order of AgB1 > AgB4 > AgB2 > AgB5 > AgB3. It was found that in some cases antibodies against subunits of AgB was not produced. In another study, Jiang *et al*[43] reported that AgB1 has higher diagnostic sensitivity in comparison with AgB2 and AgB4. However, in Virginio *et al*[44] study, antigen B8/2 provided the highest diagnostic sensitivity (93.1%) and specificity (99.5%) in ELISA system. In Leggatt *et al*[45] study, a sensitivity of 90.9% was reported for the 12 kDa subunit of AgB (corresponding to the smallest

Table 1 Performance of hydatid cyst fluid in diagnosis of cystic echinococcosis in different serological assays

Antigen	No. of subjects			Test	Sensitivity (%)	Specificity (%)	Cross reactions	Year	Ref.
	CE patients	Other disease	Healthy control						
SHCF	78	24	15	IgG ELISA	72.4		NR	2001	[71]
BHCF	129	65	203	IgG ELISA	77.6	96.6	Cysts, Toxoc.	2003	[44]
CHCF	26	35	10	Dipstick assay	100	91.4	Cysts, AE, Trichinosis, Schist., Fascio	2004	[6]
CHCF	26	35	10	EITB	100	91.4	Cysts, AE, trichinosis, Schist, Fascio	2004	[6]
CHCF	26	30	10	IgG ELISA	96.2	100	None	2004	[6]
SHCF	102	68	95	IgG ELISA	88.2	80.9	AE, Cysts, Schist, Fascio, Taeniasis, Dirofilariasis	2008	[72]
SHCF	120			Casoni's skin test	88.2	80.9	NR	2005	[73]
SHCF	120			Casoni's skin test	70	87	NR	2005	[73]
SHCF	120			Casoni's skin test	62	85	NR	2005	[73]
SHCF	120			IHA	56	84	NR	2005	[73]
SHCF	25	15	25	ELISA on serum	72	76	Cysts, Ascaris, Ambs liver abscess	2007	[74]
SHCF	25	15	25	ELISA on urine	84	76	Cysts, Ascaris, Ambs liver abscess	2007	[74]
SHCF	25	15	25	ELISA on saliva	56	76	Cysts, Ascaris, Ambs liver abscess	2007	[74]
SHCF	40	40	70	CCIEP	97.5	58.1	Fascio, Toxoc, Taenia, Malignancies	2007	[10]
SHFF	204	53	90	IEP	31	100	None	2000	[75]
SHFF	204	53	90	IHA	54	100	None	2000	[75]
SHFF	204	53	90	IB	80	96	Cysts, Serous cysts	2000	[75]
SHCF	35	12	25	Dot-ELISA	100	89.1	Ascaris, Taenia, Strogyl	2010	[1]
SHCF	35	12	25	CCIEP	80	62	Ascaris, Strogyl, Toxop	2010	[1]
SHCF	59	60	39	IgG ELISA	91.5	96	Clonorchiasis	2013	[76]
hHCF	50	15	20	IB	83	98	None	2014	[28]
hHCF	50	15	20	IgG IB	83	98	None	2014	[28]
SHCF	50	40	20	IgG ELISA	92	85	Ascaris, Ambs, Malignancy, Toxop	2014	[67]
SHCF	50	40	20	IgM ELISA	70	93.33	Ascaris, Ambs, Malignancy, Toxop	2014	[67]
SHCF	50	40	20	IgE ELISA	86	96.66	Ascaris, Ambs, Malignancy, Toxop	2014	[67]
SHCF	50	40	20	IgG1 ELISA	82	98.33	Ascaris, Ambs, Malignancy, Toxop	2014	[67]
SHCF	50	40	20	IgG2 ELISA	74	95	Ascaris, Ambs, Malignancy, Toxop	2014	[67]
SHCF	50	40	20	IgG3 ELISA	52	36	Ascaris, Ambs, Malignancy, Toxop	2014	[67]
SHCF	50	40	20	IgG4 ELISA	86	28	Ascaris, Ambs, Malignancy, Toxop	2014	[67]
Psx Ag	113	112	121	DIGFA	87.6	90.90	Hd, Cysts, HCC, HH	2015	[48]
CPsx extract	147	88	60	IgG ELISA	90	57	AE, Trypanosomiasis	2002	[76]
Emwl Ag	50	154		WB IgG	98		NCC	2000	[14]
Emwl Ag	50	154		IHA > 80	94.3		NR	2000	[14]
Emwl Ag	50	154		IHA > 320	80		NR	2000	[14]
Emwl Ag	50	154		IgG ELISA	79.4		NR	2000	[14]

EITB: Enzyme linked immunoelectrotransfer blot; IHA: Immune hemagglutination assay; CHCF: Camel hydatid cyst fluid; SHCF: Sheep hydatid cyst fluid; hHCF: Human hydatid cyst fluid; SHFF: Sheep hydatid fluid fraction; Hd.: Hepatic distomiasis; Emwl Ag: Whole larval antigen from *Echinococcus multilocularis*; CPsx extract: Crude protoescolex extract; Psx Ag: Protoscoleces antigen; BHCF: Bovine hydatid cyst fluid; Ascaris: Ascariasis; Toxop: Toxoplasmosis; Fascio: Fascioliasis; Cysts: Cysticercosis; Ambs: Amebiasis; Toxoc: Toxocariasis; Schist: Schistosomiasis; AE: Alveolar echinococcosis; Strongyl: Strongyloidiasis; HH: Hepatic hemangioma; NR: Not reported; CE: Cystic echinococcosis; ELISA: Enzyme-linked immunosorbent assay.

subunit of AgB) in a blotting system. More than 5% (5.5%) of cysticercosis patients reacted with this subunit.

The 12 kDa subunit of AgB, was cloned and expressed by Abdi *et al*[46]. The antigen was comparatively evaluated for diagnosis of CE, with native AgB and HCF. The sensitivity and specificity of rAgB, in ELISA system was similar to HCF (96% and 97%), and lower than native AgB (98.6% and 100%).

A recombinant antigen of B8/1 (rAgB), showed a high sensitivity (94.6%) and specificity (93.9%) for diagnosis of CE, using serum samples from Iran, China and Japan, in comparison with HCF, native AgB, prepared from sheep HCF, either from Iran of Japan[31].

Mamuti *et al*[41] cloned and produced recombinants of EmAgB8/1 from *E. multilocularis* and EgAgB/1 from

Table 2 Performances of antigen 5 for immunodiagnosis of cystic echinococcosis in different serological assays

	No. of subjects			Test	Sensitivity (%)	Specificity (%)	Cross reaction	Year	Ref.
	CE patient	Other disease	Healthy control						
Ag5	39	51	29	IgG ELISA	54	89	AE	2000	[9]
Ag5	58	36	40	IgG ELISA	100	70.17	Leish, Toxop, Fascio	2006	[39]
Ag5	58	36	40	IgG1 ELISA	100	70.17	Leish, Toxop, Fascio	2006	[38]
Ag5	58	36	40	IgG4 ELISA	75.8	93.02	Toxop, Fascio	2006	[38]
Ag5	58	36	40	IgE ELISA	70.1	100	None	2006	[38]
rAg5	34	36	18	IgG ELISA	65	89	AE, Cysts	2005	[77]
rAg5-38s	34	36	18	IgG ELISA	21	97	AE	2005	[77]

Leish: Leishmaniasis; Toxop: Toxoplasmosis; Fascio: Fascioliasis; Cysts: Cysticercosis; AE: Alveolar echinococcosis.

E. granulosus and evaluated their antigenic reactivity in Western Blotting and ELISA in comparison with that of counterpart, an 8 kDa subunit of AgB. WB showed reactivity with 81.3% of sera from CE patients and 40.6% of sera from alveolar echinococcosis (AE) patients, while EgAgB8/1 showed reactivity with 86% of CE and 42% of AE patients. Both EmAgB/1 and EgAgB/1 showed similar reactivity with 37.8% of sera from AE and 88% of sera from CE patients.

A synthetic P176 peptide related to N-terminal extreme of AgB/1 subunit yielded a sensitivity and specificity of 78.69 and 96.88 for pulmonary hydatid cyst[47].

Application of antigen B in a dot immunogold filtration assay increased the test specificity (98.3%) but in turn decreased the sensitivity (77.9%) of the assay, compared to native antigen[48].

Source of antigen B is an important factor which affects the performance of the test for diagnosis of CE. In agreement with this, Rahimi *et al*[49] showed that AgB isolated from human and sheep liver cyst have the best performance in diagnosis of CE when compared with those antigen obtained from liver or lungs cyst of goat, cattle or camel.

Combination of antigen B and antigen 5 may increase the sensitivity of the test as currently used in a commercially available test. The commercially available Rapid Immunochromatography test VIRapid® HYDATIDOSIS test (Vircell, Spain) using antigen 5/B was evaluated by Tamer *et al*[50] for diagnosis of CE where they reported a sensitivity of 96.8% and specificity of 87.5%. In their study, the antigen cross reacted with sera from taeniasis and leishmaniasis patients and also a few (4%) of healthy controls.

Nature and quality of antigen B, isolated from HCF, may be variable based on the host species, cyst location, cyst status and also parasite strain. This is one of the reasons that different laboratories attain different results using AgB in serodiagnosis of CE. In view of this point, discrepancies in results of serodiagnosis of CE, using antigen B might be related to, method of antigen preparation, variation in host and strain of parasite, differences in antigen B, site of the cyst, clinical status and type of the cyst. Table 3 shows the performances of antigen B in diagnosis of CE in different serological assays.

Protoscolices antigens: Native metacestode-derived antigens show substantial (mainly more than 90%) sensitivities in diagnosis of CE[51]. However cross-reactivity with other parasitic diseases (fascioliasis, schistosomiasis, amebiasis, taeniasis, cysticercosis and filariasis) is the main drawback of using such antigens for serodiagnosis of CE. The best performance for serological tests of ELISA, IHA and IFA, was achieved for ELISA (87.5% sensitivity and 100% specificity), using metacestode antigen[52].

Detection of IgG subclasses: Detecting of specific antibodies of IgG subclasses may improve the diagnostic performance of immunodiagnostic tests. Xu *et al*[53] examined the seroreactivity of 42 IgG negative (total IgG) with IgM, IgE, IgA, and IgG subclasses and found that 32 cases were positive with either one or combined of two of other antibodies. The best seropositivity (42.95%) was reported with either IgG1 alone or a combination of IgG1 + IgA + IgM. IgG subclasses is usually linked to the status of cyst development. Findings of Daeki *et al*[54] demonstrated that IgG antibody response is associated with the growth and development of cyst, while IgG1, 2 and 3 responses are predominantly related to involutive phase in CE cysts. Patients with relapsing disease have a high level of IgG4 titer.

Lawn *et al*[55] demonstrated that concentration of CE-specific IgG subclasses (IgG1-4), are much correlated with disease activity than total IgG. Among the IgG subclasses, IgG2 provided the best correlation with clinical outcome. In a lateral fellow dipstick test, a sensitivity of 95% and specificity of 100% was reported for detection of IgG4, in comparison with IgG dipstick with 87.5% specificity[56]. Detection of antibodies mainly IgG subclasses (IgG1, 4) in urine of CE patients provide a similar result in comparison to serum sample in Chirag study[57].

Antigen detection for immunodiagnosis of CE

Antigen detection has been used for diagnosis of a few of parasitic diseases with satisfactory results[58-61]. Antigen detection might be useful for detection of current infection and also post treatment follow up of CE patients. However results with detection of hydatid cyst antigen

Table 3 Performances of antigen B in diagnosis of cystic echinococcosis

Antigen	No. of subjects			Test	Sensitivity (%)	Specificity (%)	Cross reactions	Year	Ref.
	CE patients	Other disease	Healthy control						
nAg B	204	21	90	IB	66	100	None	2000	[75]
nAg B	59	55	15	IgG ELISA	80	77	AE, NCC	2005	[77]
nAg B	90	86	27	IgG ELISA	77	85	AE, RA	2000	[9]
nAg B	204	21	90	IgG ELISA	74	100	None	2000	[75]
nAg B	31	87	29	IgG ELISA	77.41	81.9	AE, Ev, Schist, Toxoc	2000	[78]
nAg B	78	24	15	IgG ELISA	93.5	89.7	Distomatosis, Schist	2001	[71]
nAg B	129	65	203	IgG ELISA	60.3	92.6	Cysts,Toxoc	2003	[44]
nAg B	22	12	4	WB	77	100	Toxoc, Other cestodes	2010	[30]
nAg B	40	40	70	IgG ELISA	92.5	97.3	Fascio	2007	[10]
nAg B	40	40	70	CCIEP	97.5	58.2	Fascio, Toxoc, Taenia, Malignancy	2007	[10]
nAg B	204	53	90	IB	66	100	None	2000	[75]
nAg B	204	53	90	IgG ELISA	74	100	None	2000	[75]
nAg B	35	29	25	IgG ELISA	94.2	81.6	NR	2009	[16]
nAg B	55	72	50	IgG ELISA	96.4	97.2	None	2014	[68]
nAg B	113	112	121	DIGFA	92.9	81	HD, Cysts, HCC, HH	2015	[48]
Goat liver Ag B	47	30	40	IgG ELISA	91.4	92.8	NR	2011	[49]
Human liver Ag B	47	30	40	IgG ELISA	97.8	97.1	NR	2011	[49]
Bovine lung Ag B	47	30	40	IgG ELISA	78.7	85.7	NR	2011	[49]
Sheep lung Ag B	47	30	40	IgG ELISA	93.6	88.5	NR	2011	[49]
Camel lung Ag B	47	30	40	IgG ELISA	93.6	90	NR	2011	[49]
Sheep liver Ag B	47	30	40	IgG ELISA	95.7	92.8	NR	201	[49]
rAgB	204	21	90	IB	72	100	None	2000	[75]
rAgB	113	112	121	DIGFA	77.9	98.3	None	2015	[48]
rAgB8/1	31	87	29	IgG ELISA	54.84	80.17	AE, Schist, Toxoc	2000	[78]
rAgB8/1	129	65	203	IgG4 ELISA	91.4	91.7	Cysts	2003	[44]
rAgB8/1	59	55	15	IgG ELISA	68	88	AE, NCC	2005	[80]
rAgB8/2	31	87	29	IgG ELISA	83.87	98.28	Schist, Toxoc	2000	[78]
rAgB8/2	129	65	203	IgG ELISA	93.1	99.5	Cysts, Toxoc	2003	[45]
rAgB8/2	129	65	203	IgG4 ELISA	69	87.5	Cysts	2003	[45]
rAgB8/2	59	55	15	IgG ELISA	45	86	AE, NCC	2005	[77]
rAgB8/1	129	65	203	IgG4 ELISA	91.4	91.7	Cysts	2003	[44]
rAgB8/1	59	55	15	IgG ELISA	68	88	AE, NCC	2005	[77]
rAgB8/2	31	87	29	IgG ELISA	83.87	98.28	Schist, Toxoc	2000	[78]
rAgB8/2	129	65	203	IgG ELISA	93.1	99.5	Cysts, Toxoc	2003	[44]
rAgB8/2	129	65	203	IgG4 ELISA	69	87.5	Cysts	2003	[44]
rAgB8/2	59	55	15	IgG ELISA	45	86	AE, NCC	2005	[77]
B1t	102	68	95	IgG ELISA	83.3	87.5	AE, Schist, Cysts, Fascio,	2008	[9]
B2t	102	68	95	IgG ELISA	91.2	93	Cysts, Schist, Fascio	2008	[9]
2B2t	186	174	110	IgG ELISA	87.6	99.1	AE, NCC, Hepatitis	2012	[9]
rEgAFFPt	129	65	203	IgG ELISA	58.6	95.6	Cysts, Toxoc	2003	[44]
rEgCaBP2	129	65	203	IgG ELISA	84.5	96.6	Cysts, Toxoc	2003	[44]
rEgcMDH	129	65	203	IgG ELISA	89.7	95.1	Cysts	2003	[44]
rEgAFFPf	129	65	203	IgG ELISA	69	89.7	Cysts, Toxoc	2003	[44]
rEpC1-GST	324	502	70	IgG IB	92.2	95.6	AE, NCC, Schist, Liver cancer	2003	[79]
rTPxEg	100	218	20	IgG IB	39	69.3	AE, NCC	2004	[78]
rEgG5	23	138	20	IgG IB	61	70	AE, Cysts	2004	[79]
E14t	102	68	95	IgG ELISA	35.3	91.7	Schist	2008	[9]
C317	102	68	95	IgG ELISA	58.8	80.9	AE, Cysts, Taeniasis, Schist,	2008	[9]
p65	90	86	27	IgG ELISA	44	96	AE, Schist, Toxoc	2000	[9]
p175	90	86	27	IgG ELISA	49	94	AE, Schist, Toxoc	2000	[9]
p176	90	86	27	IgG ELISA	80	93	AE, Schist, Toxoc, Syph, Chagas	2000	[9]
p177	90	86	27	IgG ELISA	38	92	AE, Toxoc, Syph, Chagas	2000	[9]
pGu4	90	86	27	IgG ELISA	18	98	AE	2000	[9]

Leish: Leishmaniasis; Toxop: Toxoplasmosis; Fascio: Fascioliasis; Cysts: Cysticercosis; Ascaris: Ascariasis; Syph: Syphilis; Ambs: Amebiasis; Toxoc: Toxocariasis; Schist: Schistosomiasis; NCC: Neurocysticercosis; AE: Alveolar echinococcosis; nAg B: Native antigen B; rAgB: Recombinant antigen B; Ev: Ev: Polycistic hydatid disease (*E. vogeli*); RA: Rheumatoid arthritis; HCC: Hepatocellular carcinoma; NR: Not reported; CE: Cystic echinococcosis; ELISA: Enzyme-linked immunosorbent assay.

Table 4 Performances of antigen detection assays in immunodiagnosis of cystic echinococcosis

Antigen	No. of subjects			Test	Sensitivity (%)	Specificity (%)	Cross reaction (%)	Year	Ref.
	CE patient	Other disease	Healthy control						
Urinary antigen	40	24	25	Co-A	50	89.09	12.5	2000	[62]
Serum antigen	40	24	25	Co-A	73.08	94.23	12.5	2000	[62]
Serum antigen	35	29	25	IgG ELISA	25.7	98	3.4	2009	[16]
Serum antigen	141	25	25	LAT	72	98	4	2003	[7]
Serum antigen	40	24	25	CIEP	45	100	None	1997	[80]
Urinary antigen (ucon)	40	24	25	CIEP	22.5	95.91	8.33	1997	[80]
Urinary antigen (con)	40	24	25	CIEP	47.5	95.91	None	1997	[80]

ucon: Unconcentrated; con: Concentrated; CE: Cystic echinococcosis; LAT: Latex agglutination test; Co-A: Coagglutination; CIEP: Countercurrent immunoelectrophoresis; ELISA: Enzyme-linked immunosorbent assay.

for detection of CE are far from satisfaction[7,11,62-64].

Antigen detection in CE is much less sensitive than antibody detection and the later remains the most commonly used approach for diagnosis of this disease. Antigen can be detected in sera of 35%-85% of CE patients depends on the status and location of the cyst[7,16,63]. In some cases of CE circulating antigen has been detected in sera of patients who had not shown anti-hydatid antibodies in their serum. Swarna *et al*[11] reported a sensitivity of only 53.33% and specificity of 96.66% in a Dot-ELISA system for detection of hydatid cyst antigen in urine samples. Lower sensitivity (29.68) was obtained when CCIEP was used for detection of hydatid urinary antigen[62].

Using coagglutination test, a sensitivity of 47.5% was achieved for detection of hydatid antigen in urine[63]. Several interfering factors have been proposed to explain the poor performance of antigen-detection assays in diagnosis of CE. Among them are formation of immune complexes and low availability of free antigen, sequestration of antigen due to cyst layers, especially in intact cyst, and presence of interfering component in serum or urine, as demonstrated in other studies[58]. Cysts in privileged sites (*e.g.,* eye and brain) do not release enough antigens to be detected by serological assays.

Location of the cyst is an important issue in diagnosis of CE as one study pointed out that CE antigen can be detected in 46% of patients with liver cyst but not in any of patients with lung hydatid cyst[16]. In an attempt to develop an antigen detection assay for diagnosis of CE, Sadjjadi *et al*[16] evaluated an ELISA system for detection of circulation antigens in serum of CE patients. In their study, antigen was detected in only 9 out of 35 (25.7%) of cases. Table 4 summarizes the performance of antigen detection assays in diagnosis of CE.

Post treatment follow up: CE patients need to be followed up after treatment, to make sure about the risk of recurrence. Anti-CE antibodies may persist for several years after treatment[55]. Although antigen detection might be a useful approach in post-treatment follow-up, however its low sensitivity hampered its use for patients' follow-up. Different serological assays have been used for monitoring of surgically or chemically treated CE patients[26,64-66].

In a recent cohort study, CE patients were followed for a mean of 6 years and the level and isotypes of antibodies were evaluated before and after surgical or anti-helminthic drugs treatment. Results demonstrated that IgE, IgG1 and IgG4 are the most important antibodies for serological diagnosis of active CE. During post-operation, IgM, IgE, IgG1, IgG2 and IgG4 were the best correlative with disease activities[67]. Reiterová *et al*[68] reported that antibodies to AgB was not detectable three months after treatment but antibodies to HCF were remained detectable.

It has been reported that subclasses of IgG have different performance in diagnosis of primary in comparison to relapse cases of CE. One study suggested IgG2 as a good marker for primary infection and total IgG for detection of relapse cases[69].

Recombinant P29 protein of *E. granulosus* was synthesized by Ben Nouir *et al*[64] and evaluated for post-surgical follow-up of CE patients, in an ELISA and WB systems. Results indicated that, using P29-ELISA, all of initially seropositive cases of CE seroconverted to negative within three years after treatments, while HCF-ELISA remained positive in 90% of cases. Western Blotting, using P29, remained positive in only 10% of cases after 3 years while HCF-WB remained positive in more than 25% of cases after 3 years of follow-up. However the performance of P29 in initial diagnosis of CE has not been satisfactory.

In another study by this group, somatic protoscolex antigens of *E. granulosus* have been assessed for follow-up of surgically treated CE patients and found that only 29% of treated patients reaching seronegativity after 5 years of follow up. The conventional HCF-ELISA becoming negative in 15% of cases at the end of the follow up period[65].

A double 27 and 28 kDa antigen, in WB, was also reported as useful antigen for the follow up of CE

patients. However such bands were only detectable in 75% of the patients before treatment[65]. The prognostic value of AgB subunits was evaluated by Ben Nouir *et al*[64] in ELISA and WB systems. Patients were grouped into either cured or non-cured CE patient. Findings of the study showed that ELISA remained positive 4-5 years after treatment in 57.1% of cured and 100% of non-cured patients. Immunoblotting, based on AgB subunits (8 and 16 kDa), revealed 14.3% of seropositivity after 4 years, with no reactivity to the components after 5 years of follow up. Interestingly, WB remained positive in 100% of non-cured patients up to 5 years (end of follow-up period). Serum antibodies to a certain bands (24 and 39) of HCF in Western blotting decreased in post-surgical monitoring of CE patients[70].

CONCLUSION

The performances of currently available immunodiagnostic test in diagnosis of CE are not satisfactory and the best serological test for diagnosis of CE is still the subject of debate. Over the time, particularly during the last two decades, several immunodiagnostic tests have been developed, mainly based on HCF, AgB and Ag5, yet their performance in diagnosis of human hydatidosis are unsatisfactory.

The most widely used antigens for serological diagnosis of CE are AgB and Ag5. Yet new antigens are being constantly evaluated and new serological assays are being developed to improve the performance of serological diagnostic tests.

Utilizing of recombinant or synthetic antigen although improved the performance, but has not overcome the problem of low sensitivity or even cross reactivity with other antigen in diagnosis of CE and these problems still remained. Considerable variation in performance of serological test for diagnosis of CE between different laboratories is mainly related to lack of standardization of antigen preparation, inadequate sensitivities and specificity, and also strain of the parasite that antigens have been purified from its content.

Immunodiagnostic tests based on recombinant antigen has drawn the attention of many researchers and the outcomes of such studies are promising. These antigens, especially based on AgB subunits, showed not all the times, but in most cases, satisfactory performance in comparison to their homologues antigens.

New interesting perspective in the development of serological assays for diagnosis of CE might be derive from recent observation that IgG subclasses are good markers for diagnosis and also follow up of CE patients. Moreover, the evaluation of highly purified Ag5 for immunodiagnosis of CE seems to be a promising task ahead which must be undertaken in the future. And finally immunodiagnosis assays may well be improved through combining of several well-defined antigens, notably immunodominant antigen in different stages of the cyst development.

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MINIREVIEWS

Selecting the best strategy of treatment in newly diagnosed advanced-stage ovarian cancer patients

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Abstract

Although it is assumed that the combination of chemotherapy and radical surgery should be indicated in all newly diagnosed advanced-stage ovarian cancer patients, one of the main raised questions is how to select the best strategy of initial treatment in this group of patients, neoadjuvant chemotherapy followed by interval debulking surgery or primary debulking surgery followed by adjuvant chemotherapy. The selection criteria to offer one strategy over the other as well as a stepwise patient selection for initial treatment are described. Selecting the best strategy of treatment in newly diagnosed advanced stage ovarian cancer patients is a multifactorial and multidisciplinary decision. Several factors should be taken into consideration: (1) the disease factor, related to the extension and localization of the disease as well as tumor biology; (2) the patient factor, associated with patient age, poor performance status, and co-morbidities; and (3) institutional infrastructure factor, related to the lack of prolonged operative time, an appropriate surgical armamentarium, as well as well-equipped intensive care units with well-trained personnel.

Key words: Ovarian cancer; Advanced stage; Primary debulking surgery; Neoadjuvant chemotherapy; Patients' selection

Core tip: Selecting the best strategy of treatment in newly diagnosed advanced-stage ovarian cancer patients is a multifactorial and multidisciplinary decision. Surgeries performed by gynecologic oncologists with formal training in cytoreductive techniques at referral centers are crucial factors in obtaining better oncologic outcomes. However, other factors such as clinical status of the patients, the hospital's infrastructure and equipment, as well as the tumor biology of each individual patient should also be taken into account before deciding on an initial strategy of treatment in women with advanced-stage ovarian cancer.

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INTRODUCTION

It is estimated that over 80% of women with ovarian cancer are diagnosed at advanced stages, when the disease is already extended in the abdominal cavity or beyond. Primary complete debulking surgery (PDS) followed by adjuvant chemotherapy is associated with the best oncological outcome and is considered, therefore, the standard of care[1]. Limitations, however, have been postulated with respect to this treatment strategy. First, patients with postoperative residual disease have no meaningful impact on overall survival (OS)[2-4]. Second, only in few cases is the complete primary cytoreduction rate acceptable, and only when the procedure is performed by experienced surgeons with extended formal training in cytoreductive techniques. Third, PDS is associated with a high incidence of postoperative complications[5,6].

Consequently, an alternative strategy based on neoadjuvant chemotherapy followed by interval debulking surgery (NACT-IDS) has been proposed[7]. Patients receive three to four courses of platinum-taxanes NACT and then, in the absence of progression disease, IDS is performed. The proposed advantages include a reduced risk of peri-operative morbidity, a higher rate of complete tumor resection, and a contention that deferring the initial attempt at surgical debulking does not compromise survival[8].

Nevertheless, there currently exist several controversies regarding the best strategy of treatment[9,10]. Although it is assumed that the combination of chemotherapy and radical surgery should be indicated in all newly diagnosed advanced-stage ovarian cancer patients, one of the main questions raised is how to select the best strategy for initial treatment in this group of patients, a topic that will be the focus of this review.

STEPWISE PATIENT SELECTION FOR NACT OR PDS

An algorithm of management for newly suspected or diagnosed advanced-stage ovarian cancer patients has been developed at the Instituto Valenciano de Oncologia (IVO), Valencia, Spain. Hence, patients are initially evaluated with computer tomography (CT) of thorax-abdomen and pelvis, plus tumor markers including CA-125, CA-19.9, and CEA.

Pre-operative tumor markers can also provide additional information to allow discrimination between an ovarian or extra-ovarian origin of peritoneal carcinomatosis. In this case, if the CA-125 (UI/mL)/CEA (ng/mL) ratio is < 25, mammography and endoscopy are mandatory to exclude primary breast, gastric, or colon cancer[11] (Figure 1). The main aim of the pre-operative CT scan is to localize intra-abdominal disease at non-resectable structures such as liver hilium, celiac trunk, superior mesenteric artery, supra-renal lymph node metastases, and intrahepatic metastases; as well as to identify extra-abdominal disease.

In cases of non-resectable disease at CT scan or in patients older than 75, with poor status or surgical contraindications, a core biopsy or a diagnostic laparoscopy with biopsy is performed to obtain a tissue sample. If the final diagnosis confirms an epithelial ovarian carcinoma, NACT is indicated. According to our series, a total of 30% of patients would receive NACT at this point. In the absence of the previously mentioned criteria, a mini-laparotomy is performed to rule out any extended small bowel carcinomatosis and mesentery roof retraction. These findings are present in 10% of cases, a tumor biopsy is performed and NACT is started in these patients a few days later (Figure 1). The remaining 90% of our patients undergo complete PDS, following the surgical step-wise description detailed elsewhere[12].

Patients undergoing NACT receive 3 courses of carboplatin/paclitaxel intravenously every 3 wk, and are then evaluated by using clinical examination, CA-125 and CT scan. Women with a partial response undergo IDS in an attempt to complete tumor resection. Second-line chemotherapy or inclusion in clinical trials is proposed to women with stable disease or progression to NACT.

DISCUSSION

Role of primary complete cytoreduction

Complete resection of all macroscopic disease at primary debulking surgery is the single most important independent prognostic factor in women with advanced ovarian cancer[2,4-6,13-15]. The definition of "optimal" cytoreduction has been the subject of debate for decades. Therefore, optimal residual disease, such as that measuring 1-2 cm in diameter, has been traditionally considered[16]. However, a significant improvement in survival after complete tumor resection at the time of primary surgical cytoreduction has been observed[2-4]. Thus, according to the last Gynecological Cancer Inter Group (GCIG) consensus conference, cytoreduction should be classified as "complete", without residual disease"; or "incomplete", if residual disease is left at the end of the surgery. In addition, the consensus established that the aim of surgical debulking should be to obtain a complete tumor resection[1]. The final decision as to whether or not to perform a tumor debulking depends on the surgeon's training and confidence in the majority of her or his operations on patients[17]. A great body of evidence suggests that patients operated on by gynecologic oncologists with formal training in cytoreductive techniques are more likely to undergo a complete cytoreduction in comparison to those treated by general gynecologists or general surgeons, with significantly better oncologic outcome[18,19]. Therefore, the main worldwide

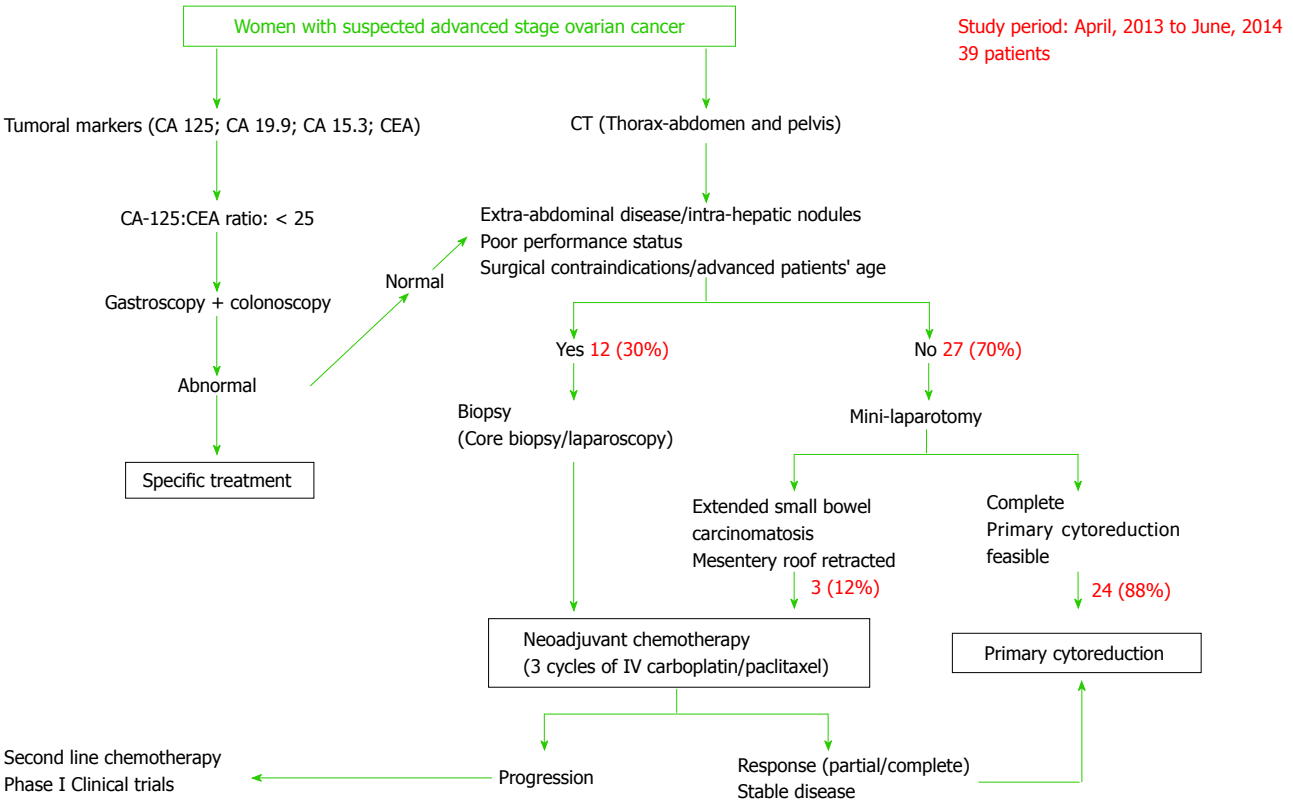

Figure 1 Stepwise management of women with suspected advanced-stage ovarian cancer. CT: Computer tomography.

consensus[1,12] states that gynecologic oncologists should make the decision regarding whether to start treatment with PDS or NACT in patients with advanced-stage ovarian cancer.

The decision regarding the initial strategy of treatment, based on NACT or PDS, in women with advanced ovarian cancer has been largely debated. A large meta-analysis involving 6885 patients in 53 studies after PDS demonstrated that each 10% increase in cytoreduction correlated with a 5.5% increase in median survival time. Patients with 75% or greater maximal cytoreductive efforts had a median survival of 37 mo compared with a 23 mo for patients with 25% or less maximal effort[14]. On the other hand, Bristow *et al*[20] studied 835 patients in 22 cohorts with advanced ovarian cancer who received NACT. The study showed a median OS of 24.5 mo, range 10-42 mo. Despite the fact that this rate was shorter than what was obtained after PDS, this comparison should be taken with caution given that a bias upon the selection of patients to receive NACT might exist. On the basis regarding the extension of the disease or performance status, within patients who underwent NACT might have a worse prognosis.

The results of the first randomized controlled trial (RCT) in patients with ovarian cancer FIGO stage ⅢC-Ⅳ of the European Organization for Treatment and Research (EORTC) and the National Cancer Institute of Canada, comparing PDS *vs* NACT-IDS, were published in 2010[11]. The authors randomized 718 patients with stage ⅢC-Ⅳ ovarian cancer, excluding ⅢC by node metastases only. Surgical time was 180 min in both arms and the median OS and progression-free survival was 30 mo and 12 mo, respectively, in the two arms. One of the main criticisms of the EORTC trial was, however, that NACT was compared to a weak PDS arm. The study was conducted in non-selected centers, achieving a median OS of 30 mo, with a complete cytoreduction rate in the PDS arm of 21%. A similar RCT performed in 87 hospitals in the United Kingdom and New Zealand found the same results[21]. It is interesting to note that these rates are markedly inferior to the outcomes reported by other international multicenter studies[2-4]. When surgery is performed at referral oncologic centers by well-trained surgeons, the complete primary cytoreduction rate can be over 40%-50%, with a median 5-year OS of 50-60 mo[2,5,6].

Despite the fact that the radicalness of the surgery is the most important factor to obtain a better oncologic outcome, other issues should also be taken into account. These factors include: (1) the time since the first visit of the patient to the commencement of the treatment; (2) the time from the hospital discharge after primary or interval debulking surgery to the initiation of adjuvant chemotherapy. Median time should not exceed 40 d, a longer period of time is related with a high incidence of postoperative complications; (3) the number of cycles in relation to neoadjuvant chemotherapy, should not be more than four; and (4) the time from the end of neoadjuvant chemotherapy to interval debulking surgery.

Table 1 Factors associated with cytoreduction rate

Factor	Characteristic
Surgeon	Adequate skills and training in cytoreductive techniques
Disease	Extension and localization of the disease Tumor biology
Patient	Advanced age Comorbidities Poor performance status
Institutional infrastructure	Ovarian cancer multidisciplinary surgical team Availability of prolonged operative time Appropriate surgical armamentarium Well-trained ICU personnel Well-equipped ICU capability

ICU: Intensive care unit.

Whether tumor biology or maximal up-front cytoreduction surgery is the most important determinant for better outcomes is being largely debated. At same time that some studies found cytoreduction removal of visible disease had a more significant impact on survival than the extent of the disease before surgery[22], other studies observed opposite results[23]. Thus, other factors should be taken into consideration in an attempt to classify ovarian tumors as with "bad" or "good" prognosis. Recent molecular studies, using microarray analysis, have associated overall survival with gene expression profiles in ovarian cancer patients after up-front surgical treatment[24]. Although future large analysis should confirm these findings, it should be expected that molecular studies using genes and proteomic pattern might represent the tools to select patients for the best individual treatment rather than to generalize one strategy over the other for all women with ovarian cancer.

However, beyond the surgeon factor previously described, the cytoreduction rate is also associated to other variables such as: (1) the disease factor, related to the extension and localization of the disease as well as tumor biology; (2) the patient factor, associated with patient age, poor performance status, and co-morbidities; and (3) institutional infrastructure factor, related to the lack of prolonged operative time, an appropriate surgical armamentarium, as well as well-equipped Intensive Care Units with well-trained personnel[12,25,26] (Table 1). It is crucial, moreover, to establish an adequate ovarian cancer multidisciplinary surgical team that includes other specialists such as general surgeons, anesthesiologists, infectologists, *etc*. (Figure 2).

Pre-operative evaluation in women with suspected advanced stage ovarian cancer

CT scan is recommended as the most appropriate imaging test prior to treatment planning in women with a suspected advanced stage ovarian cancer[27]. However, limitations with CT scan have been associated with its inability to accurately predict extensive serosal and mesenteric disease[28], and as it was previously described, these anatomical localizations are major limits to obtaining a complete cytoreduction. In fact, several models were developed to predict suboptimal cytoreduction by using CT scan parameters, but with very poor outcome[28-30].

Clinical studies have also evaluated the role of positron emission tomography (PET) and PET/CT as part of pre-operative evaluation in women with advanced-stage ovarian cancer[31]. However, based on the available literature, there is still no evidence that PET or PET/CT works better than CT in detecting the extension of primary ovarian cancer[31].

Diffusion-weighted magnetic resonance imaging (DW-MRI) is another tool under investigation used to predict resectability in women with advanced-stage ovarian cancer. The evidence for using the DW-MRI in improving detection of the true extent of the disease seems promising. The utility of DW-MRI in predicting intra-abdominal spreading in women with ovarian cancer has recently been evaluated in some investigations[32-34]. A recent study of 32 patients with ovarian cancer found the main gains of using DW-MRI were the detection of bowel serosal and mesenteric disease, with an accuracy for detection of peritoneal disease of 91% on DW-MRI compared with 75% on CT and 71% on FDG-PET/CT[34]. The results of this technique do appear to be promising for improving the detection of small volume-diffuse peritoneal disease. This encouraged data from a small number of studies; however, it should be prospectively evaluated and validated with a larger sample of patients to establish stronger conclusions in this regard.

Additional clinical factors can help surgeons to identify high-risk patients with postoperative complications and mortality after primary cytoreductive surgery. Two studies tried to correlate clinical factors with increased risk for postoperative morbidity after primary cytoreduction[35,36]. The studies observed for those aged over 75 together with either FIGO stage Ⅲ or Ⅳ and coincident comorbidity[35]; or aged over 75 combined with serum albumin < 3 g/dL or ASA score of at least 3 and high initial tumor burden (FIGO IV or high volume FIGO ⅢC)[36] identifies a subgroup of 7%-12% of patients with advanced ovarian cancer where upfront debulking surgery is associated with unacceptably high rates of morbidity and peri-operative mortality.

Laparoscopy vs laparotomy to evaluate the intra-abdominal extension of the disease

The majority of women receive either NACT or PDS based upon tumor extension and on estimated tumor resectability[17]. As has been previously detailed, there is no current imaging tool that can predict complete cytoreduction in women with advanced-stage ovarian cancer. Therefore, a direct laparoscopic or laparotomic assessment of the abdominal cavity is sometimes needed.

A pre-treatment laparoscopic score to predict tumor

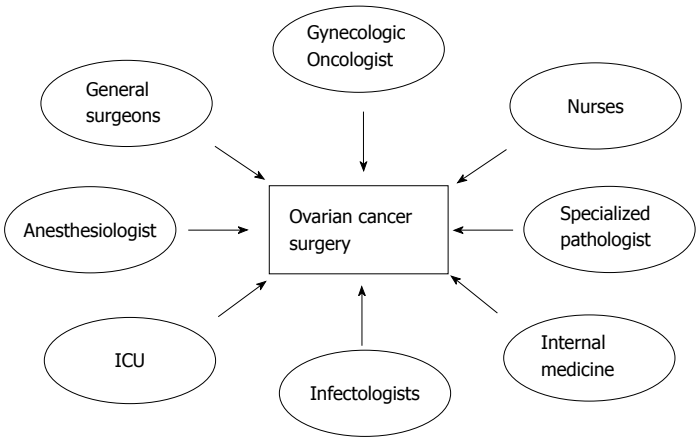

Figure 2 Multidisciplinary surgical team for treating women with advanced-stage ovarian cancer. ICU: Intensive care unit.

resectability was developed at a referral Italian cancer center[37]. This model established a predictive index value (PIV) with punctuation between 0-2 if tumors were present or not in specific areas of the pelvis and the abdomen. A score of 2 corresponded when the parameters were present, and score of 0 when they were absent. The study found that a predictive index value $\geqslant$ 8 resulted in a predictive probability of cytoreduction to less than 1 cm of zero (specificity of 100%), thus, avoiding unnecessary laparotomies. The PIV of the laparoscopic evaluation was then validated at 4 Italian Satellite Centers[38] and, more recently, the prognostic value of the laparoscopy-based-score was also established[39]. However, despite the fact that this strategy seems to be promising, some open questions still need to be clarified before its implementation into clinical practice: (1) the definition of each item is subjective, including terms such as "Unresectable massive peritoneal involvement plus milliary pattern of distribution" or "Obvious neoplastic involvement of the gastric wall"; (2) the oncologic impact of the missed assessment of the retroperitoneum is unknown; and (3) the model does not take into consideration clinical factors such as age, performance status or comorbidity. There are currently three ongoing trials which will probably answer some of these questions[40].

By using our algorithm, the evaluation of complete resectability is performed by a periumbilical longitudinal 10-cm mini-laparotomy instead of laparoscopy. By this approach, a surgeon's hand can be introduced into the abdominal cavity to carefully determine the extension of the disease on the liver surface, abdominal wall, hilium of the spleen and pancreatic tail, as well as the anterior stomach surface. In addition, this maneuver allows palpation of the most critical area of unresectability, such as liver hilium, celiac trunk, the mesenterium and the small bowel surface. This is a 40-min intervention with very low morbidity, allowing patients to start NACT 10-15 d later.

Surgical steps to obtain complete tumor resection

At our center, if complete tumor resectability is possible at the time of mini-laparotomy, patients undergo an immediate xipho-pubic midline incision with full exposure of the abomino-pelvic organs in order to establish the true extent of the disease. In this sense, before starting the removal of the disease, a stepwise systematic evaluation of the abdominal cavity is performed in order to avoid the so-called "point of no return" with unnecessary patient morbidity[12]. This standardized strategy has been well described previously[12], and includes two points of stop-or-go decisions. Initially, the *ligamentum falciforme* is resected, and the peritoneum of the paracolic gutters and the omentum are dissected from the transverse colon. Then, the lesser sac is opened allowing the evaluation of the pancreas, the *truncus coeliacus*, the liver, and the hepatoduodenal ligament with portal vein, hepatic artery and *ductus choledochus*. If a non-resectable tumor is present, surgery is stopped. If not, the second point of decision is the evaluation of the *radix mesenterii* and the small bowel surface by dissecting the adhesions and separating the small bowel from the colon and the greater omentum[12].

CENTRALIZATION OF CARE

Surgical training plays a crucial role in treating women with advanced-stage ovarian cancer[18]. Given the complexity of surgical procedures in obtaining a complete primary cytoreduction, as well as its positive impact on OS, not surprisingly, many studies from several countries have shown better OS when ovarian cancer patients were initially operated by a gynecologic oncologist rather than general gynecologist[41-43] or general surgeon[44].

Several authors have proposed the centralization of care of ovarian cancer[14,18,42,45] as an approach for improving the quality of care and outcomes. The main demonstrated benefits include better optimal cytoreduction rate[42,45], better chemotherapeutic administration rate and schemes[44,45], and better overall quality of treatment; therefore, improving the patient's quality of life. Thus, in comparison with unspecialized hospitals, patients who receive care at specialized centers may prolong their OS by almost a year[19,45]. Nevertheless, despite these clear advantages and according to population-based studies, fewer than 40% of patients with ovarian cancer have access to a specialized center in developed countries[43,44]. More recently, a study-population performed in California,

United States, demonstrated that only 4% of women with advanced-stage ovarian cancer were operated on by high-volume physicians at high-volume teaching hospitals[19].

CONCLUSION

Selecting the best strategy for treatment in newly diagnosed advanced-stage ovarian cancer patients is a multifactorial and multidisciplinary decision. Surgeries performed by gynecologic oncologists with formal training in cytoreductive techniques at referral centers are crucial factors in obtaining better oncologic outcomes. However, other factors such as clinical status of the patients, hospital infrastructure and equipment, as well as tumor biology of each individual patient should also be taken into account before deciding on an initial strategy of treatment in women with advanced-stage ovarian cancer.

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MINIREVIEWS

Helicobacter pylori and allergy: Update of research

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Author contributions: Daugule I conceived and designed the review, made data analysis and interpretation and wrote the paper; Zavoronkova J made data acquisition and classification; Santare D drafted the article, and made critical revisions.

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Abstract

Recently a lot of literature has been published about the possible preventive action of *Helicobacter pylori* (*H. pylori*) against allergy. The present review summarizes research data about the association between *H. pylori* and allergic diseases, as well as discusses possible hypotheses about the preventive action of *H. pylori* against atopy. There is evidence from observational studies to support a weak inverse association between prevalence of *H. pylori* infection and allergy. However, confounders like some unidentified socioeconomic factors, antibiotic use and others could bias the association. Although data from cohort studies point to a possible association of *H. pylori* with some of the allergic diseases, no definite proof for causal relationship has been clearly demonstrated yet. A biological mechanism proposed to explain the preventive action of *H. pylori* to allergy is reduced exposure to a major stimulus for the generation of Treg cells in individuals without *H. pylori* infection. In addition, *H. pylori* could be an indicator for changes in gut microbiome, reflecting the complex interaction between microbes and immune system.

Key words: *Helicobacter pylori*; Allergy; Atopy

Core tip: Review summarizes research data about the association between *Helicobacter pylori* (*H. pylori*) and allergic diseases. Results from observational studies support a weak inverse association between prevalence of *H. pylori* and allergy. However, different confounders like unidentified socioeconomic factors, antibiotic use and others could bias the observed association. Further, no definite proof for causal relationship has been clearly demonstrated yet, although data from cohort studies point to a possible association of *H. pylori* with some of the allergic diseases. Finally, microbiological studies show that *H. pylori* could be an indicator for changes in gut microbiome during recent decades, reflecting the complex interaction between microbes and immune system.

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INTRODUCTION

Although *Helicobacter pylori* (*H. pylori*) infection is supposed to be associated with gastric and duodenal

ulcer, gastric adenocarcinoma, MALT lymphoma and even recognized as grade-1 carcinogen[1-4], only minority of infected patients will develop a serious disease. Moreover, some researchers even suggest possible preventive effect of *H. pylori* against several diseases like gastro-esophageal reflux disease and Barret's adenocarcinoma, obesity, autoimmune diseases, allergy and others[5,6].

The latest Maastricht consensus states that the evidence available shows no definite causative protective effect of *H. pylori* against asthma and atopy nor that its eradication causes or worsens them and further research is needed[7]. Thus, new data appear constantly about the possible role of bacterium in the development of allergic diseases. The present review summarizes research data about the association between *H. pylori* and allergic diseases.

EVIDENCE FOR OPPOSITE PREVALENCE TREND BETWEEN *H. PYLORI* AND ALLERGIC DISEASES

The idea about a possible protective role of *H. pylori* against allergy has arisen observing opposite prevalence trends between *H. pylori* and allergic diseases, showing that the prevalence of *H. pylori* in industrialized countries is decreasing while the prevalence of asthma and other allergic diseases is increasing[6].

Ironically, the first case-control studies showed a positive association between *H. pylori* infection and allergy. For example, in 1998 a study from Italy identified higher prevalence of *H. pylori* IgG antibodies among allergic patients compared to patients with inflammatory bowel disease[8]. Further, Figura *et al*[9] identified higher prevalence of anti-CagA antibodies in *H. pylori* infected persons with food allergy compared to controls (62.5% *vs* 28.0%, respectively; $P = 0.03$). In addition, the mean IgE level to the most common alimentary antigens was increased in CagA-positive individuals compared to CagA-negative patients. This made the authors suggest that mucosal and inflammatory lesions found in individuals infected with CagA-positive *H. pylori* strains could increase the epithelial permeability and promote the passage of allergens, which, in atopic persons, could directly stimulate an IgE response.

Higher prevalence of *H. pylori* among patients with allergic diseases observed in some studies raised question about the role of inflammation (observed in the presence of *H. pylori* infection) in the development of allergy. A positive association between *H. pylori* and allergic diseases is still being discussed in respect to urticaria. Moreover, International guidelines in Urticaria state that in some cases of chronic spontaneous urticaria eradication of infections, such as *H. pylori*, bowel parasites and bacterial infections of the nasopharynx, have shown to provide a benefit in the management of the disease[10].

One of the first well designed and controlled studies showing opposite prevalence trends between *H. pylori* and atopy comes from Finland, demonstrating 3.5-fold increase of total and allergen-specific IgE antibody level in random population from 1973 to 1994 (OR = 5.12, 95%CI: 2.32-11.3)[11]. However, increase of IgE was observed mainly in the subgroup with no *H. pylori* antibodies, thus raising the hypothesis that *H. pylori* could influence the development of atopic diseases[11].

A huge study from United States based on database containing information about asthma and *H. pylori* status in 7663 subjects showed an inverse association between cag-positive *H. pylori* strain and asthma (OR = 0.79, 95%CI: 0.63-0.99), with a stronger association in younger individuals[12]. An inverse association was found also with other allergic disorders (allergic rhinitis and sensitization to different allergens). Further, the authors tested the association in children up till 20 years of age ($n = 7412$) and again found inverse association with wheezing, allergic rhinitis and eczema[13].

Up to now many cross-sectional and case-control studies have been performed, thus, showing controversial results. Results are summarized in Tables 1 and 2.

Basing on the published studies, several meta-analyses have tested the association. Although in 2012 Wang *et al*[14] demonstrated no association between asthma and *H. pylori* infection, analyzing five studies with 770 cases and 785 controls (OR = 1.1, 95%CI: 0.82-1.24), another meta-analysis by the same authors showed pooled OR of all included studies (nine cross-sectional, seven case-control and three cohort studies) for asthma and *H. pylori* to be 0.81 (95%CI: 0.72-0.91); while pooled OR for asthma and *H. pylori* infection in cross-sectional studies was 0.84 (95%CI: 0.74-0.96), in case-control studies - 0.94 (95%CI:79-1.12)[15].

Similarly, Zhou *et al*[16] analyzing 14 studies with 28283 persons demonstrated a weak inverse association between *H. pylori* and asthma 0.84 (95%CI: 0.73-0.96). Taye *et al*[17] have performed meta-analysis of 16 studies ($n = 21348$) about the association of atopy with *H. pylori.* The authors found an inverse association with atopy (OR = 0.82, 95%CI: 0.73-0.91) as well as with increased level of specific IgE (OR = 0.75, 95%CI: 0.62-0.92).

Detailed overview about case-control and cross-sectional studies, as well as meta-analysis of studies, has been recently published by Lionetti *et al*[18]. The authors concluded that pooled results of case-control studies showed a significant inverse association of *H. pylori* infection with atopy/allergic disease (or with atopy, but not with allergic disease), while pooled results of cross-sectional studies showed only a significant association between allergic disease and *H. pylori* infection.

However, the analysis and comparison of studies is complicated and should be evaluated with caution due to differences among study designs. The authors of the meta-analysis argue that different diagnostic criteria for allergic disease and atopy are used - in some studies asthma was diagnosed by physician tests or by symptoms, while in others the authors evaluated self-reported disease or used only laboratory tests[18].

Table 1 Association between *Helicobacter pylori* and allergy in cross-sectional studies

Ref.	Country	Studied population	*n*	Age (yr)	*H. pylori* detection	Allergy diagnosis	Main finding: OR (95%CI) in relation to *H. pylori*
Lee *et al*[35]	South Korea	Routine check-up	3376	Adults	IgG	Physician diagnosed allergy; use of anti-allergic medication; IgE	No association with allergic disease: 1.05 (0.86-1.28); Inverse association with IgE hypersensitivity: 1.32 (0.98-1.31)
Zevit *et al*[46]	Israel	National referral laboratory	6959	5-18	UBT	Physician diagnosed asthma; use of anti-allergic medication	Inverse association with asthma: 0.82 (0.69-0.08)
Imamura *et al*[47]	Japan	Healthy volunteers	211	Adults	IgG	Specific IgE, polinosis symptoms	Inverse association with polinosis: 0.15 (0.05-0.48)
Fullerton *et al*[21]	United Kingdom	General population	2437	Adults	IgG	Symptoms of wheeze, hay fever; lung function tests; bronchial reactivity; SPT; IgE	No association with asthma: 1.09 (0.77-1.54), atopy: 0.92 (0.74-1.15); hay fever: 1.00 (0.79-1.26), wheeze: 0.94 (0.74-1.19)
Pfefferle *et al*[48]	Germany	Employees of two companies	500	Adults	SAT	Self-reported physician diagnosed allergy, use of anti-allergic medication specific skin sensitization	Inverse association with allergy diagnosis: 0.26 (0.08-0.84)
Chen *et al*[13]	United States	Data from health and nutrition examination survey	7412	3-19	IgG; CagA	Self-reported asthma	Inverse association with asthma: 0.69 (0.45-1.06) Subgroup < 5 yr: 0.58 (0.38-0.88); 3-13 yr: 0.14 (0.24-0.69)
Shiotani *et al*[49]	Japan	University students	1953	Adults	IgG	Self-reported atopic dermatitis, asthma, allergic rhinitis, urticaria	Inverse association with allergic diseases: 0.49 (0.27-0.89)
Baccioglu *et al*[50]	Turkey	Patients with upper gastrointestinal endoscopy	90	Adults	Gastric tissue microscopy	SPT, total IgE, questionnaire	No association with allergic disease: 1.0 (0.1-18.9) Inverse association with asthma/CagA$^+$ cases: 0.79 (0.63-0.99);
Chen *et al*[12]	United States	Data from health and nutrition examination survey	7663	Adults	CagA	Self-reported asthma, allergen-specific skin sensitization	Inverse association with allergic rhinitis/CagA$^+$: 0.77 (0.62-0.96)
Herbarth *et al*[51]	Germany	School starters	3347	5-7	UBT	Eczema	Inverse association with eczema: OR 0.31
Kolho *et al*[52]	Finland	Patients with upper gastrointestinal endoscopy	97	5-15	Histology data	Specific IgE	No association with IgE, asthma, hay fever;
Jarvis *et al*[20]	United Kingdom	Health service registry	1121	Adults	IgG	Specific IgE, symptoms	Inverse association with sensitization to grass: 0.65 (0.43-0.99)
Uter *et al*[53]	Germany	University students	1368	18-20	IgG	Physician diagnosed asthma	No association with asthma: 0.99 (0.57-1.64)
McCune *et al*[54]	United Kingdom	Community-based population	3244	Adults	UBT	Use of asthma medication	Inverse association with asthma: 0.78 (0.59-1.05)

H. pylori: *Helicobacter pylori*; UBT: 13C-urea breath test; SAT: Stool antigen test; SPT: Skin prick test.

Further, difference between detection of active infection by urea breath test or stool antigen test and detection of *H. pylori* antibodies should be noted. In addition, the age of study population should also be taken into account since the time between *H. pylori* colonization and allergen sensitization is difficult to evaluate, therefore *H. pylori* negative adult patient could have been colonized since childhood and *vice versa*[18].

Therefore we could conclude, that, although evidence from observational studies show an inverse association between allergic disease and *H. pylori,* the association is weak and not consistent.

IS *H. PYLORI* TRULY INDEPENDENTLY INVERSELY ASSOCIATED WITH ALLERGIC DISEASE?

The idea about the inverse association of *H. pylori* with allergic diseases has been strongly criticized arguing, that *H. pylori* could be merely a marker of socio-economic status, known to be also associated with allergic disease[19].

Although Blaser *et al*[6] report, that the inverse association between *H. pylori* and asthma was observed independent of socioeconomic status, age, gender,

Table 2 Association between *Helicobacter pylori* and allergy in case-control studies

Ref.	Country	Cases (*n*)	Controls (*n*)	Age (yr)	*H. pylori* detection	Allergy diagnosis	Main finding: OR (95%CI) in relation to *H. pylori*$^+$
Pedullà *et al*[55]	Italy	Food allergy + atopic dermatitis (88)	Atopic dermatitis (202)	2-11.8	IgG, SAT	Physician diagnosed food allergy, IgE	Inverse association with food allergy: 0.32 (0.11-0.95)
Elitsur *et al*[56]	United States	Eosinophil esophagitis (62)	Esophagitis (268); idiopathic gastritis (480)	Children	Histology data	Upper endoscopy, histology data	Inverse association with eosinophil esophagitis: 0.096 (013-0.72)
Karimi *et al*[57]	Iran	Asthma (98)	Healthy children (98)	6-12	UBT	Physician diagnosed asthma	No association with asthma: *H. pylori* positivity 18% (cases) *vs* 23% (controls)
Reibman *et al*[58]	United States	Asthma (318)	Non-asthma controls (208)	Adults	IgG, CagA	Physician diagnosed asthma; IgE; spirometry	Inverse association with asthma for CagA$^+$ cases: 0.57 (0.36-0.89)
Konturek *et al*[59]	Germany	Food allergy (42)	Healthy controls (20)	Adults	UBT, IgG	Physician diagnosed food allergy; SPT, IgE, N-tele-methylhistamine urinary excretion	Inverse association with food allergy: *H. pylori* positivity 33% (cases) *vs* 40%(controls)
Annagür *et al*[60]	Turkey	Asthma (79)	Healthy children (36)	5-15	IgM and IgG	Pulmonary function tests, SPT, total IgE	No association with asthma: 1.69 (0.62-4.67)
Jaber *et al*[61]	Sauda Arabia	Asthma (220)	Asymptomatic children (543)	1-10	IgG	Physician diagnosed asthma	Inverse association with asthma: 0.84 (0.56-1.25)
Jun *et al*[62]	Japan	Asthma (46)	Peptic ulcer patients (48) + healthy controls (48)	Adults	IgG, CagA	Physician diagnosed asthma	No association with asthma: 1.20 (0.53-2.72)
Pessi *et al*[63]	Finland	Asthma (245)	Matched controls (405)	Adults	IgG	Physician diagnosed asthma	Inverse association with asthma: 0.86 (0.63-1.19)
Bartuzi *et al*[64]	Poland	Food allergy with GI symptoms (110)	Chronic gastritis (40)	Adults	Biopsy, histology	Physician diagnosed food allergy, IgE	In atopic patients *H. pylori* increases intensity of gastric inflammation
Tsang *et al*[65]	China	Asthma (90)	Healthy controls (97)	Adults	IgG	Physician diagnosed asthma	No association with asthma: 1.55 (0.87-2.78)
Corrado *et al*[66]	Italy	Atopic dermatitis (30) + atopic dermatitis with GI symptoms (30)	Asthma (30)	4-12	IgG; CagA	Physician diagnosed allergy	Positive association with atopic dermatitis compared to asthma: 56% and 37% (cases) *vs* 10% (controls)
Matricardi *et al*[67]	Italy	Atopic cases (240)	Non-atopic controls (240)	17-24	IgG	Physician diagnosed allergic rhinitis and asthma; IgE	Inverse association with atopy: 0.76 (0.47-1.24)
Figura *et al*[9]	Italy	Food allergy (38)	Matched controls (53)	4-12	IgG, CagA	Physician diagnosed food allergy; IgE	Positive association with food allergy in CagA$^+$ cases: 4.29
Corrado *et al*[8]	Norway	Food allergy (30) + asthma (30)	Inflammatory bowel disease (30)	5-14	IgG, CagA	Physician diagnosed food allergy and asthma	Positive association: 37% (cases) *vs* 10% controls

H. pylori: *Helicobacter pylori*; UBT: ^{13}C-urea breath test; SAT: Stool antigen test; SPT: Skin prick test; GI: Gastrointestinal.

ethnic background, smoking status, and hepatitis A infection, several studies indirectly suggest that other factors could influence the opposite prevalence trends and could play a role in the development of allergy.

For example, Jarvis *et al*[20] showed no association between presence of *H. pylori* antibodies and night cough, hay fever, wheezing within last 12 mo as well as sensitization to five allergens, after adjusting the patient sample for age, gender, area, number of siblings, social class. In addition, the authors observed a marked negative association of both hepatitis A and *H. pylori* with family size only in seropositive individuals (but not in those who were seronegative). This made authors suggest that those without either infection are likely to have grown up in hygienic environments, possibly less overcrowded and with a better diet.

Similarly, a well-designed cross sectional study from United Kingdom (also controlled for social class) could identify only lower lung function in individuals with *H. pylori* seropositivity. However, after adjustment for either height or social class the size of the association was reduced. No association was observed with wheezing, chronic bronchitis, self-reported asthma, atopy or bronchial hyper-reactivity[21].

No association with a group of infections was observed among Roma children living in poor hygienic

conditions compared to non-Roma children in Greece[22]. Although Roma children were found significantly more often seropositive for *Toxoplasma gondii*, hepatitis A, *H. pylori*, herpes simplex virus-1 (HSV-1), cytomegalovirus and Hepatitis B, no statistically significant differences were found between Roma and non-Roma children in respect to atopy or specific IgE level. Despite the higher numbers of exposure to infectious agents among Roma children, no protective effect for allergic disease development was evident. Even more, a positive association of the cumulative index of exposure to infections with atopy was found in the non-Roma children (OR = 1.38, 95%CI: 1.08-1.75) and in the total population (OR = 1.42, 95%CI: 1.11-1.83).

An interesting study on schoolchildren with similar genetic background but different socioeconomic environment (Finland and Russian Karelia) showed higher prevalence of allergen-specific IgE in Finnish children, while in Russian children higher prevalence of antibodies to *coxsackivirus B4*, *H. pylori, Toxoplasma gondi* and hepatitis A was detected. However, an inverse association between infections and prevalence of atopy was observed only in Russian Karelian children and the biggest effect was observed for enterovirus. However, the authors also hypothised that some other factors could be associated with infections in Russian but not in Finnish populations are responsible for the effect[23].

Finally, in Malaysia low *H. pylori* prevalence goes together with low prevalence of wheezing among 6-7 and 13-14 years old children (5.4% and 5.7%, respectively)[24], therefore scientists have concluded that *H. pylori* is only a marker for poor hygiene[25]. Although no study has been performed yet comparing the prevalence of *H. pylori* among patients with and without asthma in Malaysia, Raj *et al*[26] consider that available data speak against the unique role for *H. pylori* infection as a protective factor against asthma.

To summarize, there is evidence that *H. pylori* could not be independently inversely associated with allergic disease, but just reflect changes in environment and/or diet. The inverse association between prevalence of *H. pylori* infection and allergic diseases observed in studies could also be biased by some other uncontrolled factors.

POSSIBLE CAUSAL RELATIONSHIP BETWEEN *H. PYLORI* AND ALLERGIC DISEASE

Although a weak inverse association between *H. pylori* and allergy can be recognized, scientists argue that opposite prevalence, possibly evident from observational studies, does not mean a causal relationship[19]. However, demonstration of a possible causal relationship between *H. pylori* and allergy is extremely complicated.

A biological mechanism proposed to explain the preventive association of *H. pylori* to allergy is reduced exposure to a major stimulus for the generation of Treg cells in individuals without *H. pylori* infection[27]. One of the latest ideas involves neutrophil-activating protein of *H. pylori* that could inhibit Th2-mediated bronchial inflammation in patients with allergic asthma[5]. Possible immunomodulatory properties of *H. pylori* are well described by Arnold *et al*[28].

Fulfilment of Bradford Hill criteria

Blaser *et al*[6] used Bradford Hill criteria to support the evidence about the inverse association between *H. pylori* and asthma. Hill's criteria consist of several conditions fulfillment of which can provide evidence of a causal relationship between an incidence (*H. pylori* prevalence) and a possible consequence (asthma)[29].

To prove the causal link Blaser *et al*[6] mentioned the small but consistent trend demonstrated in several studies as well as the fact, that inverse causation is not likely and the decline is preceding the increase in asthma. However, although there is a weak trend showing inverse association between allergy and *H. pylori,* not all studies approve it. Further, Blaser *et al*[6] considered that the inverse association observed with early life asthma (not with long-standing asthma seen in adults) supported the role of *H. pylori,* since the effect of *H. pylori* might be less important in adult-onset asthma due to much more heterogeneous nature of adult asthma. However, confounding factors that could influence the association are not fully ruled out.

Further, one of the Bradford criteria states that there is no other likely explanation of disease - the more specific an association between a factor and an effect is, the bigger the probability of a causal relationship. However, at present allergologists consider asthma as a multifactorial disease associated with several other risk factors (like urban outdoor and indoor pollution, allergens, *etc.*) rather than *H. pylori* infection[30]. Therefore the fulfillment of Bradford Hill's criteria, demonstrated by Blaser *et al*[6], should be interpreted with caution and should be considered only as one of the arguments for protection of *H. pylori* against asthma.

Data from cohort studies

Since it is impossible to perform interventional studies to test the link between *H. pylori* and allergy, some knowledge about a possible causal association could be driven from cohort studies. However, it should be noted that such studies are not conclusive and they give only a better insight about a possible causality.

Holster *et al*[31] detected the presence of *H. pylori* antibodies in 7-9 years old children who were followed from birth and assessed by yearly questionnaires about allergic symptoms and possible risk factors. The authors observed no association between *H. pylori* and atopic dermatitis, allergic rhinitis and asthma. A borderline association was found only between *H. pylori* and wheezing. However, the authors admit, that they were not able to detect if *H. pylori* infection preceded the diagnosis of allergic disease, since presence of *H. pylori* infection was diagnosed only at the age of 7-9 years.

Further, a cohort study in Ethiopia followed children

since birth, detecting presence of allergic symptoms with questionnaires and performing allergic skin tests and *H. pylori* stool antigen tests at the age of one, there and five years. The sample was controlled for potential confounders. After three year follow-up the authors found only a borderline association with eczema[32]. Further, following the same cohort for five years an inverse association was observed only with eczema[33]. No association was observed with asthma or other allergic disease. Interestingly, that in the same cohort the association between paracetamolum therapy and allergic symptoms was analyzed separately and an inverse association was observed between use of paracetamolum and wheezing and eczema[34].

Development of allergy after H. pylori eradication

Several studies demonstrated development of an allergic disease or increase of IgE after *H. pylori* eradication. Korean study demonstrated increased levels of IgE related, non IgE related allergy as well as subclinical raise of IgE levels in patients after *H. pylori* eradication compared to *H. pylori* positive patients without eradication and *H. pylori* negative controls[35]. However, this could also be related to the change in gastric acidity due to treatment with proton pump inhibitors, used together with eradication therapy. In addition, some patients continue use of acid lowering drugs even after eradication therapy.

Data from animal studies

Finally, a possible causal relationship can be demonstrated in animal models. One of the first studies showing causal relationship was the study by Arnold *et al*[36], showing that animals infected with *H. pylori* infection had lower airway hyper-responsiveness, tissue inflammation, and goblet cell metaplasia. Further studies supported the finding that *H. pylori* infection could protect mice from development of allergic asthma[37]. However, effect observed in animal models quite often is not observed also in humans.

H. PYLORI AS A PART OF COMPLEX INTERACTION BETWEEN MICROBES AND HUMAN IMMUNE SYSTEM

H. pylori and other infectious agents

Blaser *et al*[6] speculate, that *H. pylori* could be merely a marker for other phenomena, for example, early life antibiotic use could eliminate *H. pylori* as well as other microbes that actually could be the protective agents. Therefore, the question arises if *H. pylori per se* plays the crucial role in the development of allergy or it is just a marker of frequent infections or other factors, since several other microbes have been shown to be inversely associated with allergic disease[38].

This could be indirectly supported by study, showing that seropositivity to *H. pylori* and Hepatitis A was unrelated to atopic status, while multivariate analysis showed that both the effect of having two or more younger siblings (OR = 0.1, 95%CI: 0.03-0.8) and of acquiring measles up to the age of three (OR = 0.2, CI: 0.03-0.8) were significantly related to a lower risk of asthma[39]. The finding indicates that frequent infections observed more often in families with siblings are more important than *H. pylori i*nfection *per se*. Further, Janson *et al*[40] demonstrated that combination of different infectious agents [hepatitis A, *H. pylori*, *Toxoplasmosis gondii*, HSV, Chlamydia pneumoniae, Ebstein Barr virus (EBV) and cytomegalovirus] was an independent risk factor for atopy (OR = 1.43, 95%CI: 1.06-1.93), allergic asthma (OR = 1.82, 95%CI: 1.12-2.98), and allergic rhinitis (OR = 1.69, 95%CI: 1.21-2.37).

Importance of several pathogens (*Ascaris lumbricoides, T. gondii,* HSV and EBV) for prevention of atopy has been shown in a study by Alcantara-Neves *et al*[41]: Children with three or fewer infection markers had a higher prevalence of specific IgE and skin prick test reactivity compared with those with four or more infection markers. On the contrary, isolated infections were not associated with the prevalence of atopic or non-atopic wheeze.

Therefore, evidence from studies suggests that *H. pylori* could be just a part of complex interaction between immune system and pathogens, as proposed by Janson *et al*[40].

H. pylori as a part of gut microbiome

This goes together with the idea that the increase in allergic diseases could be caused by changes in the composition of gut microflora due to global changes of environmental, socioeconomic and life style factors[27]. Data exist, showing lower diversity of microflora in allergic patients compared to healthy controls: A study in Sweden reports a lower diversity of the total microbiota at one month in infants with IgE-associated eczema[42].

Although previously *H. pylori* was considered as the major inhabitant of stomach, at present up-to-date sequence based molecular methods have allowed identifying gastric microbiota more precisely. von Rosenvinge *et al*[43] have shown that such phyla as *Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria* and *Fusobacteria* dominate in gastric fluid samples. In a review Engstrand *et al*[44] have also summarized that gastric micro-biota contains a large variety of genera including *Staphylococcus, Streptococcus*, *Pervotella, Lactobacillus* and some others, therefore *H. pylori* could be considered only a part of a complex microbial flora in the stomach.

Further, Rosenvinge has identified that treatment with proton pump inhibitors promotes bacterial overgrowth, while antibacterial treatment is associated with reduced bacterial diversity[43]. Decreased microbiota diversity in patients after *H. pylori* eradication therapy has been identified also by Jakobsson *et al*[45]. Therefore, one can conclude, that after *H. pylori* eradication the diversity of gastric microflora decreases that could possibly be

associated with development of allergy.

Therefore we could hypothise that loss of *H. pylori* results in a small (possibly significant) reduction of stimulation of immune system, as proposed by other authors[44]. Importance of other microorganisms should also be considered in the complex interaction between the human immune system and microbes. However, how *H. pylori* specifically and the entire human microbial ecosystem affect human health is still questionable.

CONCLUSION

Evidence from observational studies supports a weak inverse association between prevalence of *H. pylori* infection and allergy. However, it could be biased by confounders like socioeconomic factors, antibiotic use and others. No definite proof for causal relationship has been clearly demonstrated yet, although data from cohort studies point to a possible association of *H. pylori* with some allergic diseases. In addition, *H. pylori* could be an indicator for changes in gut microbiome during recent decades, reflecting the complex interaction between microbes and immune system.

Summarizing the data, it seems that *H. pylori* infection alone cannot prevent development of allergy in all infected individuals, similarly like bacterium is not causing a serious gastrointestinal disease in all infected patients. In both conditions genetic and environmental factors (diet, other microbes, microflora, *etc.*) are of importance next to the recognized role of the bacterium.

Nevertheless, the intensive research in *H. pylori* field has brought a new insight into the interaction between microbes and immune system and the microbial - host relationship, supporting the idea that microbes could play a role in the development of allergy.

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Monitoring anticoagulant therapy with new oral agents

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Abstract

Thromboembolic disease is a major leading cause of mortality and morbidity in industrialized countries. Currently, the management of these patients is challenging due to the availability of new drugs with proven efficacy and security compared to traditional oral vitamin K antagonists. These compounds are characterized by a predictable pharmacokinetic profile for which blood monitoring is not routinely needed. Nevertheless, some data have suggested inter-patient variability in the anticoagulant effect of these drugs, raising concerns about their effectiveness and safety. Although mass-spectrometry is the gold standard to determine drug plasma concentrations, this method is not widely available in every-day practice and some coagulation assays are commonly used to determine the anticoagulant effect of these drugs. The present review aims to summarize the current knowledge regarding the clinical question of how and when to monitor patients with new anticoagulant oral agents.

Key words: Anticoagulant agents; Apixaban; Dabigatran; Drug monitoring; Rivaroxaban

Core tip: New oral anticoagulants are replacing oral vitamin K antagonists for some practical advantages, like unnecessary monitoring and a better pharmacokinetic profile. Nevertheless, in some circumstances, their anticoagulant activity must be monitored in order to prevent adverse outcomes. In this minireview a list of the available laboratory test are reviewed to better understand the pros and cons of each analysis.

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INTRODUCTION

The management of thromboembolic disease has always been challenging since current treatments involve an inherent risk of bleeding that must be counterbalanced by the anticoagulant effect of each drug.

The use of vitamin K antagonists, such as warfarin, implies the monitoring of their anticoagulant effect through frequent blood tests and the education of patients about

Table 1 Pharmacokinetic features of new oral anticoagulants[11,12]

	Apixaban	Rivaroxaban	Dabigatran
Posology	Twice daily	Once daily	Twice daily
Oral bioavailability	45%	> 80%	6%
Half life	12 h	7-11 h	12-17 h
Excretion	25% renal	66% renal (active and inactive)	80% renal

potential drug and food interactions, making their use puzzling and difficult for many clinicians. In contrast, new agents have shown some advantages over vitamin K antagonists, since no dose adjustment and monitoring is routinely needed, as a consequence of a "more favorable pharmacokinetic profile". In addition, these new drugs, usually mentioned as new oral anticoagulants (NOACs), have demonstrated clinical efficacy and a better security profile than warfarin in various clinical trials, making attractive their use in clinical practice[1-3].

However, recent data have emerged regarding a potential role for monitoring the anticoagulant effect of these drugs particularly in patients with specific circumstances and comorbidities in order to reduce side effects and improve efficacy[4-7].

Some recommendations to measure the anticoagulant effect of NOACs include the following scenarios[8,9]: (1) Bleeding or recurrence of thrombosis; (2) Before surgery or any invasive procedure when the patient has taken the drug in the previous 24 h or longer if creatinine clearance is less than 50 mL/min; (3) Identification of supra or subtherapeutic levels in patients taking other drugs with potential interactions, or in patients with extreme body weight; (4) Patients with renal failure or prompt to it; (5) Reversal of anticoagulation; (6) Suspicion of drug overdose; (7) Patients with genetic mutations (*e.g.*, rs2244613 minor allele carriers for dabigatran etexilate); and (8) Assessment of compliance.

Although it is desirable to explore the anticoagulant effect of these drugs in the aforementioned circumstances, some cons have also emerged and detractors of routine monitoring include the following reasons in their arguments[10]: (1) Lack of measures in clinical trials; (2) Wide therapeutic window of some of these agents; (3) There is no a standardized clinical method to detect the anticoagulant effect or it is not yet available; and (4) The interpretation and dose adjustments have not been established.

PHARMACOLOGY OF NOACS

NOACs are categorized according to their site of action; apixaban and rivaroxaban act by inhibiting factor Xa, thereby decreasing the conversion of prothrombin to thrombin. On the other hand, dabigatran acts by directly inhibiting thrombin. Table 1 summarizes some pharmacokinetics features of clinical utility for these agents. Of particular interest is the renal clearance of these drugs that modifies or prohibits their use in case of severe kidney failure.

Although it is thought that there are fewer drug interactions for NOACs than for warfarin, clinical data suggest moderate to severe drug-drug interactions when dabigatran is used in combination with verapamil, amiodarone, and dronedarone[13,14].

Similarly, some other drugs commonly known as CYP inhibitors such as ketoconazole, itraconazole, macrolides, human immunodeficiency virus protease inhibitors can increase the serum NOACs concentration. On the other hand, some CYP inductors, such as phenytoin, phenobarbital, rifampicin and carbamazepine can decrease the anticoagulant effect of NOACs and thus are not generally recommended in these patients.

DETERMINING ANTICOAGULATION LEVELS WITH THE NOACS

The gold standard to measure plasma drug concentrations is mass-spectrometry. Nevertheless, the availability and laboratory expertise for doing this specialized technique is not fulfilled in the majority of clinical centers. For this reason, some other test must be carried out in order to determine the anticoagulant effect of NOACs. Table 2 resumes the advantages and drawbacks of available coagulation tests that have been used to determine the anticoagulat effect of NOACs in clinical settings.

Dabigatran

Activated partial thromboplastin time and thrombin time: These are very sensitive assays that do not accurately reflect plasma dabigatran concentrations. Although they are widely available, they are affected by a lot of variables such as inappropriate collection, improper handling and storage. Besides neither are strong predictors of bleeding, and patients may present any kind of hemorrhage even when the activated partial thromboplastin time and/or thrombin time are within normal range[15].

Diluted thrombin time: Since assays to evaluate thrombin activity are very sensitive to determine the anticoagulant effect of dabigatran, the use of diluted plasma in conjunction with the Hemoclot thrombin inhibitor permits to easily measure dabigatran anticoagulant activity[16].

Ecarin clotting time and ecarin chromogenic assay: These assays are not widely available and calibration is required to perform these tests. They use a metalloprotease called ecarin and are very specific for anti thrombin inhibitors due to the fact that prothrombin is a substrate for these analyses[17].

Rivaroxaban

Prothrombin time: The Subcommittee of Control of Anticoagulation of the Scientific and Standardization Committee recommends that this assay can determine the relative intensity of anticoagulation in patients taking

Table 2 Available coagulation tests to determine the anticoagulant effect of oral anticoagulants

Drug	Coagulation test	Pros	Cons
Dabigatran	aPTT	Highly available	Do not reflect the intensity of coagulation Low specificity
	TT	Highly available	It only determines the effect of dabigatran but lacks specificity
	dTT	Very accurate and precise to estimates plasma concentrations of dabigatran	Requires specific calibrators and controls in specialized laboratories with trained personal Low specificity
	ECT		Requires specific calibrators and controls in specialized laboratories with trained personal Limited standardization and validation required Low specificity Interlot variability reported
	ECA	Very accurate and precise to estimates plasma concentrations of dabigatran	Requires specific calibrators and controls in specialized laboratories with trained personal Low specificity
	DRVV-T		Requires specific calibrators and controls in specialized laboratories with trained personal Low specificity
Rivaroxaban	PT	Highly available	Do not reflect the intensity of coagulation Low specificity
Rivaroxaban and Apixaban	Chromogenic anti-Xa assays	Very accurate and precise to estimates plasma concentrations of dabigatran	Requires specific calibrators and controls in specialized laboratories with trained personal
	DRVV-T		Requires specific calibrators and controls in specialized laboratories with trained personal Low specificity

Any of these tests have been associated with clinical endpoints and data regarding their use in special populations are scarce. aPTT: Activated partial thromboplastin time; DRVV-T: Dilute Russell's viper venom time; dTT: Dilute thrombin time; ECA: Ecarin chromogenic assay; ECT: Ecarin clotting time; PT: Prothrombin time; TT: Thrombin time.

rivaroxaban but it is not useful to extrapolate plasma concentrations. Besides, it has different sensitivities according to the type of the employed reagent with high variability among laboratories[8,18].

Dilute Russell's viper venom time: This is a useful test to determine the anticoagulant effect of Xa and thrombin inhibitors since Russell's viper venom contains a potent activator of factor X and II. Nevertheless, validation and calibrations are technical issues that must be explored in future trials to determine a valid cut off and their sensitivity is low[19].

Chromogenic anti-Xa assay: Plasma concentrations of rivaroxaban and anti-Xa levels correlate fairly well and it is the preferred method to estimate plasma concentrations. This method is less affected by sample handling or clotting factors in patients. However, a major limitation is the standardization and the availability of laboratories with specific calibrators and controls[20].

Apixaban

Dilute Russell's viper venom time: As pointed before, this test is very useful and sensitive to determine the anticoagulant effect of apixaban, but with a low specificity.

Chromogenic anti-Xa assay: It is the most sensitive assay to determine the inhibition of factor Xa by apixaban and it is recommended to estimate plasma concentrations of this drug. Nevertheless, this test is not widely available and it is time consuming[21].

SPECIAL CONSIDERATIONS

It is important to point out that any of these analyses have been tested in special populations such as elderly patients and children, as well as pregnant women and individuals with multiple morbidities[22,23]. Besides, some methodological issues regarding sample collection must be accomplished to avoid misinterpretations and biases. For example, plasma concentrations of the NOACs can vary among 10 to 20 times between peak and trough concentrations. Therefore, the assessment of the anticoagulant activity of each drug should be obtained immediately prior to the next scheduled dose[22]. Furthermore there are few reports regarding a correlation between any of these tests and the efficacy and security of any NOAC and the clinical significance of these analyses must be interpreted cautiously.

CONCLUSION

Despite of the potentials scenarios in which the role of monitoring the anticoagulant effect of NOAC can be clinically valid, it must be point out that there is no trial which has compared results of these drugs with or without coagulation monitoring and there are no guidelines to determine the steps to follow in order to improve the quality of the anticoagulation therapy.

Nevertheless, with the broad use of NOACs in clinical practice we must keep in mind the inter-patient variability of these drugs that can result in loss of efficacy and security.

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Biomarkers of oxidative stress in erythrocytes as a function of human age

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Abstract

Despite more than 300 theories to explain the aging process, oxidative stress theory offers the best mechanism to explain aging and age related disorders. Several studies has shown the importance of oxidative stress during aging. PubMed, Science Direct and Springer online data bases are taken into consideration to write this mini-review. Human erythrocytes are most abundant and specialized cells in the body. Erythrocytes were extensively studied due to their metabolism and gas transport functions. Recent studies on erythrocytes have provided us detailed information of cell membrane and its structural organization that may help in studying the aging and age associated changes. The susceptibility of an organism is associated with the antioxidant potential of the body. Erythrocytes have potent antioxidant protection consisting of enzymatic and non-enzymatic pathways that counteract with reactive oxygen species, thus maintaining the redox regulation in the body. The non-enzymatic and enzymatic antioxidants and other biomarkers associated with erythrocyte membrane transport functions are the main content of this review. Biomarkers of oxidative stress in erythrocytes and its membrane were taken into the consideration during human aging that will be the main subject of this mini-review.

Key words: Biomarkers; Humans; Aging; Oxidative stress; Erythrocytes; Erythrocyte membrane

Core tip: The aim of present review is to summarize important oxidative stress biomarkers in erythrocytes during human aging. Erythrocyte membrane is rich in

lipids and proteins which are easy targets of reactive oxygen species. Erythrocytes are also equipped with antioxidant defense system. Studies on biomarkers of oxidative stress are important in the establishment of reference values in different populations and in studies involving their role in different disease conditions.

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INTRODUCTION

Aging is a characterized by alterations that takes place in a single cell or in the whole organ system. The exact process of aging is still not well understood but many evidences support that it is associated with excess production of free radicals in the form of reactive oxygen species (ROS) and reactive nitrogen species (RNS) throughout life[1]. During aerobic respiration, ROS/RNS are produced from electron transport chain present inside mitochondria (Table 1). Excess ROS/RNS damages proteins, lipids and nucleic acids, when enzymatic and non-enzymatic antioxidants of the body are unable to scavenge free radicals[2]. Even under normal metabolic conditions, certain amount of oxidative damage to cell and its membrane takes place, but its rate increases with the increase of OS during aging, as the antioxidant variation machinery gets diminished[3,4]. Besides many recent studies at molecular level like telomere shortening contributes to the accumulation of DNA damage during cellular aging[5], erythrocytes cell as a whole and its membrane has its own importance in aging and age associated diseases.

Human erythrocytes or red blood cells (RBCs) are in the circulatory system for 120 d[6], which transport oxygen from the lungs to all other tissues of the body and carbon dioxide (CO_2) from the body tissues back to the lungs. These erythrocytes are produced in the bone marrow by differentiation process and hematopoietic stem cells differentiate to form nucleate erythrocytes. After degradation of endoplasmic reticulum and formation of nuclei, reticulocytes appear in the circulation. An erythrocyte is a disc shaped, 8 μm biconcave structures bounded by a plasma membrane. The protein and lipid bilayer to erythrocytes changes throughout the whole life. This can be particularly seen at the stage of its plasma membrane[7], since it is made up of protein-lipid bilayer. Erythrocyte contains a conjugate protein in the form of hemoglobin. The main function of hemoglobin is the binding and releasing oxygen and carbon dioxide, for this reason the membrane of RBC is extremely important. The plasma membrane is a two dimensional meshwork of protein called as spectrin membrane skeleton. It helps in maintenance of structure of erythrocytes. Because of the above mentioned facts of erythrocyte and the plasma membrane, this cell type has been studied extensively.

Table 1 Reactive oxygen species

Oxygen centered radicals	Oxygen centered non-radicals
$O_2\cdot$	H_2O_2
$\cdot OH$	
$HOO\cdot$	$^1O_2$
$ROO\cdot$	

RBCs as oxygen carriers are continuously exposed to high oxygen tension. Oxidative stress, which decreases the antioxidant capacity, irreversibly damages erythrocytes, resulting in their eventual damage by hemolysis and their removal by circulation. Because mature RBCs are cells without nucleus and other cell organelles, they have no capacity to repair the damaged components. Celedón *et al*[8], showed that biochemical alteration which takes place during acute hypobaric hypoxia, make erythrocyte susceptible to oxidative stress. During erythrocyte life span there are many changes in size and deformability, lipid and protein content in the membrane, ion exchange and action of enzyme. This review will also address the effect of OS in erythrocytes during normal aging based on the structural alteration in the erythrocytes and its membrane. The widely studied clinical biomarkers of oxidative stress and its mechanism in human erythrocytes has been represented in Figure 1.

BIOMARKERS RELATED OF OXIDATIVE STRESS

Lipid peroxidation-malonaldehyde

Malonaldehyde, is a byproduct of lipid per-oxidation. It reacts with amino acids, membrane proteins, phosphatides, DNA and RNA of the cell, which leads to structural and functional modification. Excess level of malonaldehyde (MDA) levels has been reported when it undergoes cell damage or in several diseased conditions. Red blood cell membrane is made up of 60% of phosphatides. Cholesterol (non-esterifies) represents about 30% of the lipidic erythrocyte composition, and the last 10% are carbohydrate containing lipids[9]. Due to presence of polyunsaturated fatty acids (PUFA), cell membrane becomes more susceptible to free radicals and its oxidation takes place that breaks double bonds of PUFA and generate malonaldehyde. Previously, we have reported elevated level of erythrocyte MDA in normal elderly population[10]. Increased MDA level was supported by other findings, such as, decreased membrane sulfhydryl (-SH) groups and total antioxidant potential in older individuals compared to younger individuals[11]. The increases in the MDA level are found in diabetes, hypertension, inflammation, coronary heart and liver disease[12]. The free radical chain reaction is the mechanism involved in the process of lipid peroxidation. It increased with the increased production of ROS/RNS.

The erythrocyte membrane is rich in phosphatides

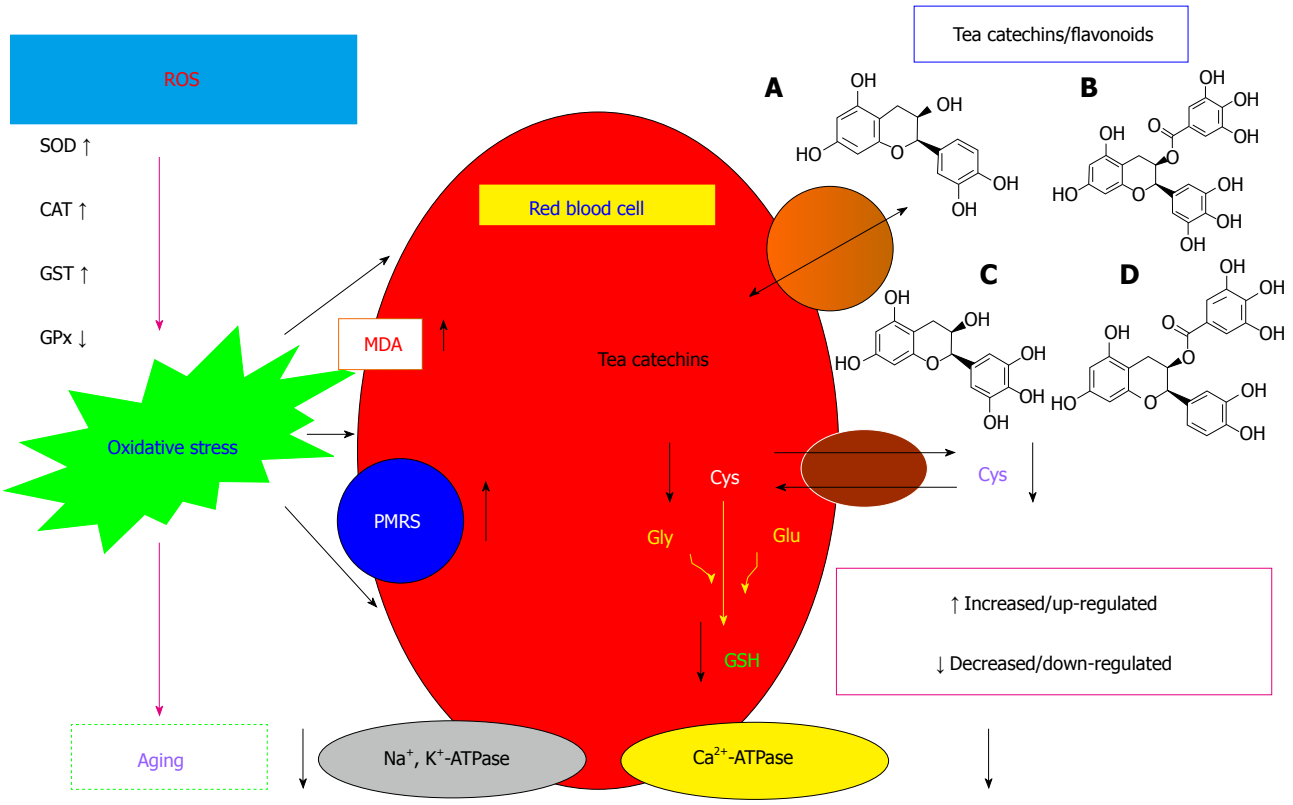

Figure 1 Aerobic cell produce reactive oxygen species as a byproduct of cellular respiration. Erythrocytes are equipped with antioxidant defense system for overcome excess production of ROS. The activity of SOD, CAT, GST and PMRS is upregulated while GPx activity is down regulated during human aging. Increased oxidative stress during aging results in elevated MDA levels and decreased GSH levels. Cysteine influx and efflux decreases during aging, which is an important amino acid for GSH biosynthesis. Red blood cell membrane bound enzymes (Na^+, K^+-ATPase; Ca^{2+}-ATPase) activity decreases as a function of human age. Dietary flavonoids like tea catechins modulate various biomarkers of oxidative stress and are protective in nature. Chemical structures of epicatechin (A), epigallocatechin gallate (B), epigallocatechin (C), and epicatechin gallate (D). Cys: Cysteine; Glu: Glutamic acid; Gly: Glycine; GSH: Reduced gluathione; GSSG: Oxidised glutathione; MDA: Malonaldehyde; SOD: Superoxide dismutase; CAT: Catalase; GST: Glutathione-S-transferase; GPx: Glutathione peroxidase; PMRS: Plasma membrane redox system; ROS: Reactive oxygen species.

containing proteins present outside the membrane. The proteins present in the outer membrane are easy target for free radicals which results in the formation of MDA. Increased MDA level results in alterations of the cell membrane polarity, charge sharing across lipid phase surface and oligomer formation. Further elevation in lipid peroxidation, results in decline resistance towards denaturation process, decreased number of membrane-SH groups and altered mobility of lipids. Several studies reported the importance of peroxidation of lipids in caloric restriction and longevity in different populations[13]. It has been observed that animals and avians have long life because they contains less number of unsaturated fatty acids in its plasma membranes, so they are not easy targets for ROS/RNS and have lower degree of MDA formation and less modification in proteins in long life of these species. Results clearly indicate that the animals/species having low degree of unsaturated fatty acids/unsaturation have longer life span.

Reduced glutathione

Reduced glutathione (GSH) is a primary antioxidant of erythrocytes. It is a tripeptide containing there amino acids and is an intracellular non-protein sulphydryl (-SH) compound. A significant decrease in erythrocyte GSH level has been reported in human erythrocytes[10]. Erythrocyte GSH level negatively correlated between decline GSH and age has been observed, this decrease is also correlates with total antioxidant potential of plasma[14]. Erythrocytes contain most important hydrophilic antioxidant in form of GSH[15]. Reduced glutathione contains sulphur containing amino acid cysteine which is the rate liming amino acid in GSH biosynthesis. Due to presence of cysteine amino acid, GSH helps in maintaining reduced status of SH group of cell membrane, including many other biological functions. OS results in the oxidized form of SH groups, which will results in many cellular and functional dysfunctions. A study carried out in European subjects also demonstrate a significant decline in erythrocyte GSH level during aging[16], while other reports on the same study did not show significant alterations in intracellular glutathione[17]. Oxidized form of glutathione (GSSG) is not favourable for body. A recent study on age associated change in the glutathione level in brain of rat shows decline in glutathione in almost all regions of the brain. The conversion of reduced glutathione to oxidized glutathione was further increased and the ratio of these two (GSH/GSSG) was

also reported to decrease, which measures the oxidation/reduction status of the cell[18]. The GSH/GSSG ratio has many biological functions including oxidation/reduction signaling and in the protection of antioxidants. The ratio also helps in providing a link to influence of environment with elderly population[19].

Membrane-SH group

The human RBCs are rich in membrane SH groups. The -SH group play a major role in maintenance of oxidation-reduction status of the cell[20]. The OS caused by ROS/RNS in erythrocytes effect cell membrane and its mechanical characteristics. Any oxidative damage to the plasma membrane-SH group of erythrocyte will induce the alterations in micro-elasticity under pathological and physiological state of OS[21]. Alterations in the oxidation/reduction balance during normal aging can modify several enzyme activates and proteins within the cell. Most of the protein molecules contain sulphur containing amino acids, methionine and cysteine which are subject to reox changes. We reported as related decrease in erythrocyte membranes-SH group during human aging[10]. Enzymes and proteins having -SH group are easy target for ROS/RNS. Age related changes in protein oxidation have been documented in erythrocytes and plasma[22]. Nitric oxide is highly reactive having half life of few seconds yet it can diffuse cell membrane freely which make NO ideal for a transient signal molecule. It is known to be involved in aging process[23].

ENZYMATIC ANTIOXIDANTS

RBCs are exposed permanently with potentially damaging level of ROS/RNS, but their metabolic processes are capable of reversing this oxidative damage under normal conditions. However, it is well known that variety of physiological and pathological factors may increase ROS/RNS which induces the oxidative stress. In addition, hemoglobin is known to stimulate lipid peroxidation[24]. Erythrocytes are equipped by antioxidant defense system in form of enzymatic and non-enzymatic antioxidants[25]. This protective system in form of enzymatic antioxidants includes superoxide dismutase (SOD)[26], which detoxify the effect of superoxide radical ($O_2^{\cdot-}$) catalase (CAT)[27], which is involved in the conversion of H_2O_2 to H_2O, and other enzymatic antioxidants like glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione-S-transferases (GSTs)[28,29]. Two enzymes are shared in H_2O_2 detoxification: CAT and GPx but their relative significance in H_2O_2 scavenging is still not clear[30]. The reduced glutathione (GSH) is a non-enzymatic antioxidant.

In human subjects there is a considerable disagreement in age-related changes of erythrocyte SOD and CAT activity[31,32]. Lower activities of CAT and SOD were shown in premature infants during first 72 h of their life in comparison with full-term infants and even during aging[33,34]. Less than 10% of normal erythrocyte CAT activity was observed in homozygous carrier of inherited CAT deficiency-acatalasemia[35], and less than 50% in heterozygous subject's hypocatalasemia[36]. An elevated SOD and CAT activities during aging in human erythrocytes has been reported[33]. Increased OS during normal aging was compensated by elevation in the activities of these enzymes. Elevated CAT and SOD activities may be a manifestation of more production of ROS/RNS during aging in humans. Several contradictory results have been shown in published reports for SOD and CAT role in normal aging process, the reason for which is hard to explain[37,38]. As far as pathological processes are concerned; decreased CAT activities were found in erythrocytes from human patients of different ages with several types of brain disorders including dementia, stroke and Parkinson disease[39,40].

Oxidative stress with alterations in profile of antioxidant enzymes in erythrocytes is also related to many others specific pathologies[41,42]. Cell requires certain enzymes to detoxify the toxicants. GSTs are group of enzymes that play a very important role in the detoxification of dangerous compounds to less toxic compounds[43]. Age associated changes in GST has been reported[44,45]. GSTs also play a significant role in drug resistant development in tumor cells, Alzheimer's and Parkinson's disease, atherosclerosis[46,47]. GST are involved in many biological functions in mammals which includes detoxification of toxicants, catalysis of several biological processes, several functions associated with metabolism, resistance towards drugs and inhibiting age associated disorders[48,49].

Age associated changes in the activity of GPx has been shown in human erythrocytes and correlated with total antioxidant capacity[50]. Several studies have been reported having conflicting data as to how GPx activity is changes with age[51]. Increased GPx activity have been shown in smaller population studies, while decreased activity has been reported in most large studies as a function of age[52]. GPx activity decreases in presence of more H_2O_2, which ultimately leads to direct cell/tissue damage and activation of nuclear factor-κB - related inflammatory pathways[53,54].

ALTERATIONS IN ERYTHROCYTE MEMBRANE TRANSPORT FUNCTIONS

In most of the eukaryotic cell membrane, the lipids are distributed in asymmetric manner across the bilayer plan. This kind of structural organization is referred as trans asymmetry. This trans-asymmetric distribution play very important role in structural and functional aspect of cell membrane. Homeostasis is very important for the cell. Transport of ion across the cell membrane are regulated by various kind of cell membrane enzymes such as Na^+, K^+-ATPase and Ca^{2+}-ATPase[55]. A recent study reports a significant decreased activity of these two enzymes during human aging[56]. Transport of dietary flavonoids across cell membrane is well documented[57]. Recently, we reported the beneficial effect of tea catechins in erythrocytes during human aging[58,59]. L-cysteine is a

sulphur containing amino acid that has free functional -SH group which is important in oxidation/reduction reactions. Free -SH group in erythrocyte membrane will help in the regulation and maintenance of intracellular redox status of erythrocytes and other cell types. Human erythrocyte does not have any machinery to synthesize protein inside the cell. Synthesis of GSH take place inside erythrocyte and it require L-cysteine, a semi-essential amino acid. GSH is a tripeptide containing glutamic acid, cysteine and glycine joined together with the help of peptide bonds. All above said amino acids are required for the biosynthesis of GSH, but the rate depends on only the availability of L-cysteine. We report L-cysteine influx and efflux across human erythrocytes during aging[60,61]. L-cysteine is the amino acid which provides free functional -SH group to GSH which play a very important role in antioxidant defense system. GSH is a soluble antioxidant which protect cell from ROS/RNS caused oxidative damage. Recently, there are substantial evidences in literature which supports that normal aging is accompanied with higher level of OS. Incorporation of flavonoids and cysteine in diet has been shown to alter several OS biomarkers which are known to be associated with aging and age related disorders[62]. We report a significant decreased efflux of L-cysteine in erythrocytes during aging[63]. Since, GSH biosynthesis in erythrocytes is dependent on L-cysteine bioavailability; the decreased influx and efflux may explain the low GSH level reported in erythrocytes as a function of age[10]. L-cysteine transported into erythrocytes only when there is properly reduced membrane lipid and protein thiol. Aging is associated with reduced antioxidant capacity which results in induction of conformational and structural changes in the cell membrane and in the amino acid transporters that finally results in L-cysteine influx and efflux.

CONCLUSION

Red blood cells are the main cell types present in blood. It is reviewed in this mini-review that erythrocytes have a very particular membrane structure and composition which alters during aging and that support their features and functions to study human aging. It has been shown that RBCs have ability to encounter various oxidative stressors to prevent oxidative stress. They are good model to study various plant products to evaluate their anti-aging properties. These findings of biomarkers of OS during normal aging will help in the establishment of reference values for biomarkers of OS in elderly peoples and several other parameters. These reference values will help in studies that involve the role of biomarkers in various other diseased conditions.

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MINIREVIEWS

Forkhead box protein A2 and T helper type 2-mediated pulmonary inflammation

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Abstract

The transcription factor forkhead box protein A2 (FOXA2, also known as hepatocyte nuclear factor 3β or transcription factor 3β), has been found to play pivotal roles in multiple phases of mammalian life, from the early development to the organofaction, and subsequently in homeostasis and metabolism in the adult. In the embryonic development period, FOXA2 is require d for the formation of the primitive node and notochord, and its absence results in embryonic lethality. Moreover, FOXA2 plays an important role not only in lung development, but also in T helper type 2 (Th2)-mediated pulmonary inflammation and goblet cell hyperplasia. In this article, the role of FOXA2 in lung development and Th2-mediated pulmonary inflammation, as well as in goblet cell hyperplasia, is reviewed. FOXA2 deletion in airway epithelium results into Th2-mediated pulmonary inflammation and goblet cell hyperplasia in developing lung. Leukotriene pathway and signal transducers and activators of transcription 6 pathway may mediate this inflammation through recruitment and activation of denditric cell during lung developments. FOXA2 is a potential treatment target for lung diseases with Th2 inflammation and goblet cell hyperplasia, such as asthma and chronic obstructive pulmonary disease.

Key words: Forkhead box protein A2; T helper type 2 inflammation; Pulmonary; Development; Goblet cell hyperplasia

Core tip: The transcription factor forkhead box protein A2 (FOXA2) plays pivotal roles in embryonic development and

organogenesis. Conditional deletion of FOXA2 in airway epithelial cells during the early stage of lung development will result in abnormal morphology of the lung and T helper type 2-mediated pulmonary inflammation. In addition, FOXA2 regulates the goblet cell differentiation during lung development and in pulmonary diseases such as asthma and chronic obstructive pulmonary disease. FOXA2 may be a new target for the treatment of lung disease.

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INTRODUCTION

The transcription factor forkhead box protein A2 (FOXA2), also known as hepatocyte nuclear factor 3β (HNF3β), is first identified by its ability to regulate liver-specific gene expression[1]. FOXA2 is a part of the large Forkhead box (*FOX*) gene family that all members have the DNA binding "winged helix domain"[2]. The gene of *FOXA2* is located in chromosome 20p11.21. FOXA2 is able to bind to specific DNA sequence[3], activate or inhibit the transcriptional activity of target genes, and also participate in cellular signal transduction[4] and metabolism regulation[5]. Meanwhile, it plays a key role in the development[6] and mature of tissues and organs[7].

With the development of mouse embryos, the first active *FOXA* gene is FOXA2 whose RNA and protein are detected on day 6.5 of gestation in the primitive streak and node[8], suggesting that FOXA2 plays an essential role in the formation of the primitive streak and endoderm[9]. The research has indicated that FOXA2 is required for the maintenance of dopaminergic properties in ventral midbrain neurons at late embryonic stage[10]. The expression of FOXA2 is also found in the liver, pancreas, lung, intestine, thyroid gland and prostate[11], implying that FOXA2 not only regulates the organogenesis and development of liver[7,12,13] and lung[14,15], but also participates in the process of glucose[16] and lipid metabolism[17]. Many studies have also shown that FOXA2 has a close relationship with the occurrence and metastasis of tumor[18-20]. For the past few years, FOXA2 is found to participate in regulating the lung development, Th2-mediated pulmonary inflammation and goblet cell hyperplasia[21,22].

STRUCTURE OF FOXA2

FOXA2 gene is located in chromosome 20p11.21 and its length is 2242 bp. As a member of the FOXA family, FOXA2 has a forkhead domain (FHD) complexed to a target DNA. The first 3D structure of FHD resolved by X-ray crystallography was that of FOXA3/HNF3γ in 1993[3]. Subsequently, the FHD structures of other members were resolved, which are similar to that of FOXA3[23], so is FOXA2. The FHD contains three N-terminal α-helices (H1-3), three β-strands and two loops (W1-2) towards its C-terminal region[24]. The recent data about the FOXA function have identified the FOXA proteins as "pioneer factors" whose binding to promoters and enhancers enable chromatin access for other factors[25-27]. It is unique in that FOXA2 is the only one in the family which contains an AKT2/PKB phosphorylation site at the N terminus of the FHD[27]. FOXA2 has two nuclear localization sequences (NLS) which are located at both ends of the FHD[28], one of the two NLS in H1 while the other in W2[24] (Figure 1).

ROLE OF FOXA2 IN DEVELOPMENT

Research has shown that the FOX superfamily express in many kinds of organism from invertebrate to vertebrate and its subfamily FOXA participants in the whole process of the embryonic development[29]. FOXA2, a member of FOXA family, is the first gene of this family to be expressed in the progress of embryogenesis[30]. In the study of mouse embryogenesis, the expression of the *FOXA2* gene appears first at the anterior of the primitive streak. After the primitive node has formed, FOXA2 expression is localized in the primitive node, notochord and neural plate[31]. Mice lacking FOXA2 die by E10 to E11 and show marked defects in structures related to embryogenesis, without forming a distinct primitive node, aberrant somites and neural tube resulting from the absence of the notochord, and failure to form the gut tube, although endoderm cells are present[6]. The defects of the notochord and neural tube, can be ascribed to a deficiency of Sonic hedgehog, as FOXA2 cooperates with the homeobox gene Goosecoid in the activation of this gene[32]. Furthermore, FOXA2 can activate the canonical WNT-β-catenin pathway and subsequently induce the primitive extraembryonic endoderm by directly up-regulating the Wnt6[33].

With the embryonic development, the expression of *FOXA2* is also detected in definitive endoderm and endoderm-derived apparatus such as liver, pancreas, and prostatic gland, where it persists through development to adulthood[30,31,33-36]. As to the lung, *FOXA2* expresses in the endoderm which later differentiates into the lung buds, where it expresses continuously in the pulmonary epithelium to adulthood[37]. *FOXA2* is found in specific subsets of respiratory epithelial cells. In the respiratory epithelium, *FOXA2* can activate the transcription of thyroid transcription factor-1 (TTF-1), clara cell secretary protein and surfactant proteins (A-D), which mark the differentiation of epithelial cells[38,39]. Moreover, the surfactant proteins A-D play critical roles in surfactant function and homeostasis[40]. In the mice lacking *FOXA2* in conducting airways, pulmonary abnormalities are not observed by light microscopy at E18.5. However, the decreased alveolar septation and peripheral saccules appear at PN3. At PN15 and later, pulmonary

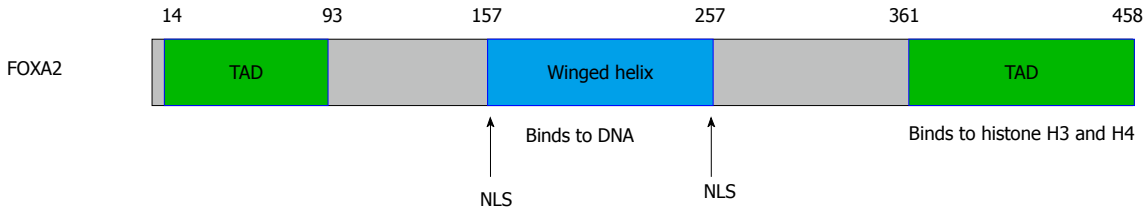

Figure 1 Structure of forkhead box protein A2 protein. FOXA2 has two transactivation domains and two nuclear localization sequences. There is a winged helix in the central of FOXA2. FOXA: Forkhead box protein A; TAD: Transcription activation domain; NLS: Nuclear localization sequences.

abnormalities including emphysema in distal airways and goblet cell hyperplasia in bronchi and bronchioles are observed in the FOXA2$^{\Delta/\Delta}$ mice[15,41]. *FOXA2* is indeed a positive regulator for E-cadherin gene[42], a cell adhesion molecule required for normal lung branching morphogenesis and cell differentiation[43,44]. Mildred *et al*[45] verified that the temporal-spatial expression patterns of *FOXA2* in the developing and regenerating of lung fit in with their proposed function in epithelial cell differentiation and regeneration, and surfactant protein gene expression. In summary, *FOXA2* plays a critical role in the development of lung.

ROLE OF FOXA2 IN TH2-MEDIATED PULMONARY INFLAMMATION AND GOBLET CELL HYPERPLASIA

Evidences showed that respiratory epithelial cells lining conducting airways regulate the inflammatory responses caused by allergens, pathogens and injurious agents[46,47]. Disruption of FOXA2 in respiratory epithelial cells results in airspace enlargement, neutrophilic pulmonary infiltrates, mucus hypersecretion, goblet cell hyperplasia and metaplasia (GCHM)[15], associated with the activation of pro-GCHM Stat6 and epidermal growth factor receptor signaling pathways[15,21,48]. Further study demonstrates that lack of FOXA2 in airway epithelial cells results in Th2-mediated pulmonary inflammation, including infiltration of eosinophiles, up-regulation of Th2 cytokines and chemokines, goblet cell hyperplasia and mucus hypersecretion, accompanied by the activation of leukotriene pathway at PN15[21]. All these findings are common in the lung of asthma patients. Therefore, FOXA2 expression in the lung may be disturbed in asthma. In fact, decreased expression of FOXA2 in the lung is found in asthma patients compared with control subjects[49]. The decreased expression of FOXA2 was negatively correlated with increased expression of mucin-5ac (MUC5ac) and chloride channel accessory 1[49]. Furthermore, the expression of FOXA2 in airway epithelial cell is inhibited by allergen challenge, and by over-expression of Th2 cytokines such as interleukin (IL)-4 and IL-13 in mouse airway epithelium[15], and also by IL-13 stimulation in human bronchial epithelial cells[48]. All these results indicate that FOXA2 plays a critical role in Th2-mediated pulmonary inflammation in developing lung. Although it inhibits goblet cell hyperplasia and metaplasia, conditional over-expression of FOXA2 in the respiratory epithelium in adult mice prior to ovalbumin (OVA) sensitization cannot alter Th2 cytokine production or inflammation in the lung[21]. Inflammatory cell counts, as well as IL-4, IL-5, IL-13, IL-10, and interferon-γ concentrations, are similar in bronchoalveolar lavage fluid (BALF) from FOXA2 over-expressing and control mice after OVA exposure[21]. Tang *et al*[22] also demonstrate that in the very early stage (from PN0 to PN10) of lung development after birth, Th2-mediated inflammation is missing in the lung of mice with FOXA2 deletion in airway epithelium. The Th2 related cytokines and chemokines are up-regulated from PN7 and Th2 inflammation in the lung is obvious on PN15. All the results indicate that Th2-mediated pulmonary inflammation induced by deletion of FOXA2 in airway epithelial cells is development-depended.

MECHANISM OF FOXA2 REGULATING TH2-MEDIATED PULMONARY INFLAMMATION AND GOBLET CELL HYPERPLASIA

The mechanism of Th2-mediated pulmonary inflammation induced by deletion of FOXA2 in airway epithelial cells remains unknown at present. Dendritic cell (DC) plays a very important role in Th2-mediated pulmonary inflammation. Chen *et al*[21] investigates the role of myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) in this inflammation. They found that the frequency of both DCs and mDC/pDC ratio are significant increased in the lung of *FOXA2*$^{\Delta/\Delta}$ mice[21]. They also found that frequencies of mDCs expressing B7-DC, B7-H1, and CD86 are significantly elevated[21]. The results indicate that increased recruitment and activation of pulmonary mDCs may mediate the Th2 inflammation in the lung in *FOXA2*$^{\Delta/\Delta}$ mice during development. However, the mechanism of the recruitment and activation of pulmonary mDCs after FOXA2 deletion in airway epithelial cells is not clear. Previous studies have provided direct evidence that cysteinyl leukotrienes (cys-LTs) plays an important role in regulating Th2 cell-dependent pulmonary inflammation[50,51]. Further study discloses that FOXA2 regulates 15-lipoxygenase (Alox15) and Alox5 gene transcription associating with leukotrienes (LTs) biosynthesis and lung inflammation[52,53]. Montelukast,

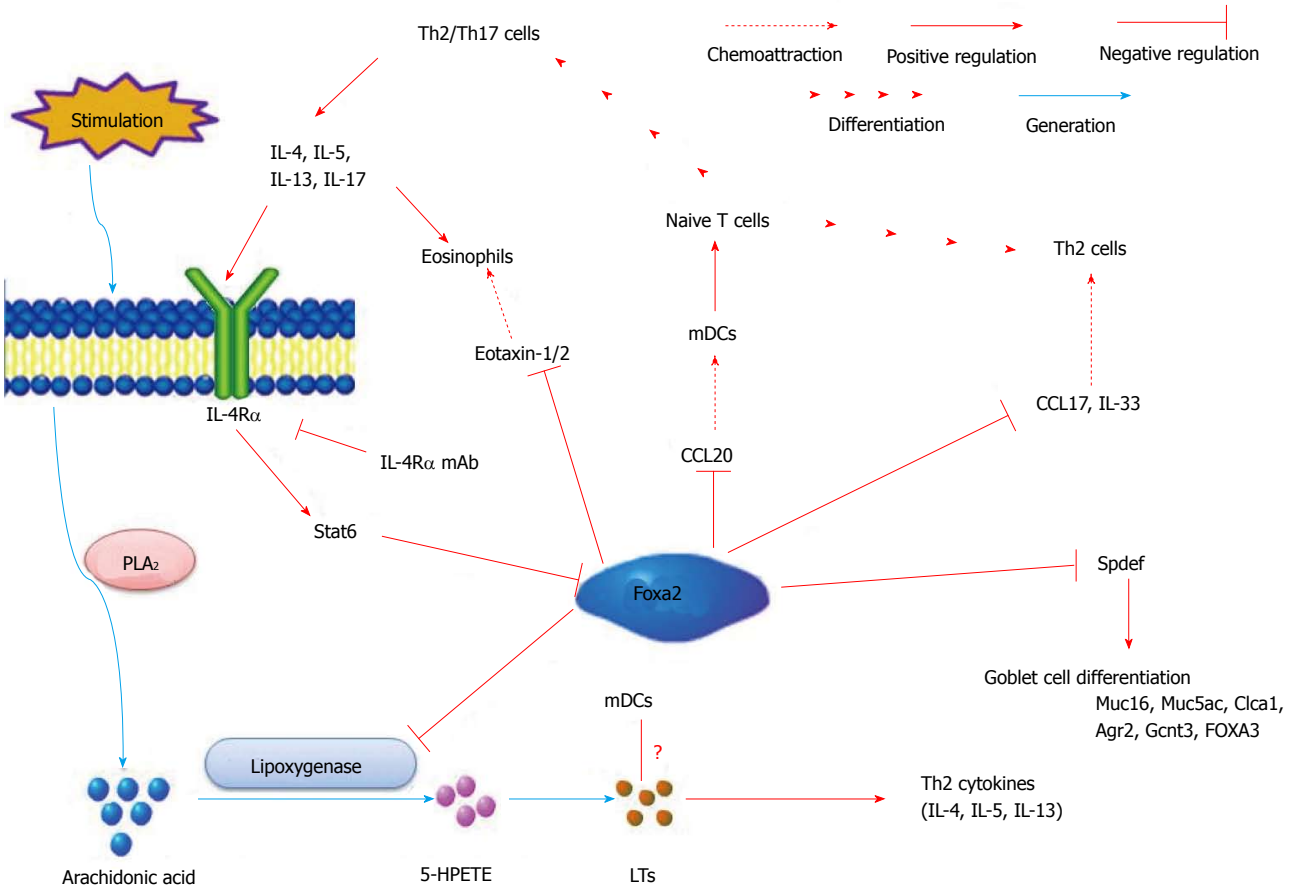

Figure 2 Network of forkhead box protein A2 regulating T helper type 2 inflammation and goblet cell hyperplasia. IL: Interleukin; mDC: Myeloid dendritic cell; CCL: Chemokine (C-C motif) ligand; Stat6: Signal transducers and activators of transcription 6; PLA2: Phosolipase A2; HPETE: Hydroperoxyeicosatetraenoic acid; LTs: Leukotrienes; MUC: Mucin; FOXA: Forkhead box protein A; Gcnt3: Glucosaminyl (N-Acetyl) transferase 3.

a selective inhibitor of the CysLT$_1$ receptor[54,55], suppresses the Th2-mediated inflammation arising from the ablation of FOXA2 in the developing mice lung. In developing FOXA2$^{\Delta/\Delta}$ mice, the increased expression of Th2 cytokines followed the activation of LT pathway. In brief, these findings uncover that FOXA2 is required for the repression of Th2-mediated pulmonary inflammation during lung development *via* its regulation to CysLT pathway[22]. Therefore, deletion of FOXA2 in the early stage of lung development leads to the spontaneous activation of LTs pathway. The activated LTs pathway may increase the recruitment and activation of pulmonary mDCs and then mediate the Th2 inflammation in the lung of *FOXA2*$^{\Delta/\Delta}$ mice during development. However, this hypothesis needs more direct evidences (Figure 2).

IL-13/IL-4-STAT6 pathway plays a critical role in Th2-mediated pulmonary inflammation[56,57]. Chen *et al*[21] tests whether Th2-mediated pulmonary inflammation and goblet cell differentiation caused by conditional deletion of FOXA2 in the airway epithelium is depend upon IL-4R–mediated signaling. The results indicate that administration of IL-4Rα mAb, an antibody which blocks IL-4Rα (a key molecular in IL-13/IL-4-STAT6 signaling pathway), significantly inhibits eosinophilic inflammation and goblet cell metaplasia and mucus hyper-production in *FOXA2*$^{\Delta/\Delta}$ mice. These results indicates that IL-4Rα-STAT6 pathway mediated the Th2 pulmonary inflammation and goblet cell hyperplasia in *FOXA2*$^{\Delta/\Delta}$ mice during lung development[21]. Wan *et al*[15] found that intratracheal administration IL-4 resulted in the decrease expression of FOXA2 and this effect is STAT6-depended. However, over-expression of FOXA2 in airway epithelium of adult mice inhibis goblet cell metaplasia and mucus hyper-production caused by OVA, but not Th2-mediated pulmonary inflammation[21]. These results indicate that the interaction between FOXA2 and IL-13/IL-4-STAT6 signaling pathway may be reciprocal in Th2-mediated inflammation and goblet cell hyperplasia in the lung.

REGULATION OF *FOXA2* EXPRESSION

FOXA2 in airway epithelial cells plays important role in lung development and Th2-mediated pulmonary inflammation, as well as in goblet cell hyperplasia. Therefore, regulation of *FOXA2* expression in airway epithelial cells may have potential role in the pathogenesis and treatment of lung diseases, such as asthma and chronic

obstructive pulmonary disease (COPD). Unfortunately, No medicine up-regulating *FOXA2* expression has been investigated in animal model or patients with COPD and asthma. Recent study indicated that Tetrapeptide Ala-Asp-Glu-Leu, a peptide which is effective on models of acute bacterial lung inflammation, fibrosis, and toxic lung damage, could increase the expression of *FOXA2* and decrease expression of MUC5ac in cultured bronchial epithelium[58]. Whether this peptide has the same effect *in vivo* has not been tested yet.

Thioredoxin-interacting protein (TXNIP) increases the expression of human islet amyloid polypeptide (IAPP) in beta-cell. TXNIP-induced FOXA2 transcription factor expression is conferring this effect by promoting FOXA2 enrichment at the proximal FOXA2 site in the IAPP promoter[59]. TXNIP can down-regulate miR-124a expression, which can directly target FOXA2. Indeed, miR-124a overexpression led to decreased FOXA2 expression and also can be effectively inhibited by TXNIP[59]. Thus, this study identifies a novel TXNIP/miR-124a/FOXA2/IAPP signaling cascade linking the critical beta-cell signaling pathway. However, whether this pathway also plays a role in airway epithelial cells and thus regulates the goblet cell hyperplasia and mucus production remains unknown.

Recent study demonstrates that over expression of NK2 homeobox 1 (NKX2-1, also known as TTF-1), inhibits allergen-induced goblet cell hyperplasia and airway inflammation[60]. Further study indicates that loss of FOXA2 in airway epithelial cell is prevented by over expression of NKX2-1 at the same time[60]. All these results suggest that NKX2-1 may regulate the FOXA2 expression in airway epithelial cell.

CONCLUSION

In conclusion, as a member of the FOX superfamily, FOXA2 participates in the formation and development of organs. Meanwhile, FOXA2 plays a very important role in lung development, Th2-mediated pulmonary inflammation and goblet cell hyperplasia. Lose of FOXA2 in the early stage of lung development will result in abnormal morphology of the lung and Th2-mediated pulmonary inflammation. FOXA2 regulates the goblet cell differentiation during lung development and in pulmonary diseases such as asthma and COPD. LTs pathway and STAT6 pathway which are regulated by FOXA2 mediate the Th2 pulmonary inflammation and goblet cell hyperplasia. Moreover, other transcription factors, such as NXK-2-1, may cooperate with FOXA2 in lung development, Th2-mediated pulmonary inflammation, and also in lung diseases with goblet cell hyperplasia.

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ORIGINAL ARTICLE

Basic Study

Recombinant outer membrane protein F-B subunit of LT protein as a prophylactic measure against *Pseudomonas aeruginosa* burn infection in mice

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Author contributions: Rasooli I contributed to the conception and design of the study, or acquisition of data, or analysis and interpretation of data; Farsani HH carried out the experimental part of the project; Astaneh SDA supervised the experiments; Nazarian S drafted the article; Gargari SLM revised it critically for important intellectual content; final approval of the version to be submitted was made by Rasooli I.

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Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Shahed University (IACUC protocol number: 917584001).

Animal care and use statement: The animals were housed in clean standard animal care facility of Shahed University. The research was carried out in compliance with the Animal Welfare Act and regulations related to experiments involving animals. The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions *viz*; 25 ℃, equal daily light/dark hours, 50% humidity and ad libitum access to food and water, for two wk prior to experimentation. The thermal injury was brought about under anaesthesia. All animals were euthanized by 150 mg/kg pentobarbital sodium for tissue collection.

Conflict-of-interest statement: The authors declare no conflict of interests.

Data sharing statement: Technical details and dataset available from the corresponding author at rasooli@shahed.ac.ir. No additional data are available.

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Abstract

AIM: To study immunogenicity of outer membrane

protein F (OprF) fused with B subunit of LT (LTB), against *Pseudomonas aeruginosa* (*P. aeruginosa*).

METHODS: The OprF, a major surface exposed outer membrane protein that is antigenically conserved in various strains of *P. aeruginosa*, is a promising immunogen against *P. aeruginosa*. In the present study recombinant OprF and OprF-LTB fusion gene was cloned, expressed and purified. BALB/c mice and rabbits were immunized using recombinant OprF and OprF-LTB and challenged at the burn site with *P. aeruginosa* lethal dose of 10^4 CFU. The protective efficacy of rabbit anti OprF IgG against *P. aeruginosa* burn infection was investigated by passive immunization.

RESULTS: It has been well established that the LTB is a powerful immunomodulator with strong adjuvant activity. LTB as a bacterial adjuvant enhanced immunogenicity of OprF and anti OprF IgG titer in serum was increased. Experimental findings showed significantly higher average survival rate in burned mice immunized with OprF-LTB than immunized with OprF or the control group. Rabbits anti OprF IgG brought about 75% survival of mice following challenge with *P. aeruginosa*. Post challenge hepatic and splenic tissues of mice group immunized with OprF-LTB had significantly lower bacterial load than those immunized with OprF or the control groups.

CONCLUSION: These results demonstrate that LTB-fused OprF might be a potential candidate protein for a prophylactic measure against *P. aeruginosa* in burn infection.

Key words: *Pseudomonas aeruginosa*; Outer membrane protein F; B subunit of LT; Immunization; Burn

Core tip: *Pseudomonas aeruginosa* (*P. aeruginosa*) is an opportunistic pathogen that infects hospitalized, burned and immunosuppressed patients. Vaccination of high-risk groups may reduce the incidence and spread of infection. In this study outer membrane protein F (OprF) and fusion genes containing OprF and B subunit of LT were cloned and expressed. The proteins were administered to the experimental mice challenged at the burn site with lethal dose of *P. aeruginosa*. Significant protection was noted in immunized animals.

Farsani HH, Rasooli I, Gargari SLM, Nazarian S, Astaneh SDA. Recombinant outer membrane protein F-B subunit of LT protein as a prophylactic measure against *Pseudomonas aeruginosa* burn infection in mice. *World J Methodol* 2015; 5(4): 230-237 Available from: URL: http://www.wjgnet.com/2222-0682/full/v5/i4/230.htm DOI: http://dx.doi.org/10.5662/wjm.v5.i4.230

INTRODUCTION

Pseudomonas aeruginosa (*P. aeruginosa*) is a major cause of morbidity and mortality in hospital acquired infections. The pathogen invades the host tissue of mainly immunocompromised patients. *This pathogen is one of the* most common organisms isolated in nosocomial pneumonia, urinary tract infection, surgical site infection, burn wounds, the cornea and the lower respiratory tract and those with the cystic fibrosis (CF)[1]. *P. aeruginosa* infecting strains are initially nonmucoid. The organism converts to mucoid and alginate producing followed by development of biofilm that enhance its antibiotic resistance[2]. The eradication of Pseudomonas frequently proves difficult due to antibiotic resistance and the ability to form a biofilm in case of chronic infection[3]. Antibiotic resistance and biofilm formation on mucosal surfaces further complicates the therapy. Hospital-derived *P. aeruginosa* strains can become colonized in burn patients that survive the initial burn trauma, are not easily eradicated with antibiotic therapy[4,5]. The initial clinical trials on *P. aeruginosa* vaccines established vaccine safety, however the limited effectiveness in preventing subsequent infection clearly evidenced the need for reevaluating correlates of vaccine efficacy[6]. Although a significant humoral response was elicited by lipopolysaccharide (LPS) vaccination, it was not able to prevent subsequent infection brought about by *P. aeruginosa*[7,8]. However, both LPS vaccines did not meet the approval for routine clinical use because of their toxicity associated with their lipid A fraction[9]. This was due in large part to the inability of the vaccine to provide protection against a broad range of *P. aeruginosa* serotypes[10]. Outer membrane proteins (OMPs), LPS and flagellin have been evaluated as vaccine candidates[11,12]. Conserved region from amino acids of flagellin and two OPMs, *i.e.*, outer membrane protein F (OprF) and OprI in clinical isolates from CF patients have been studied for their protective immunogenicity[6,13]. This sequence was used to create a vaccine preparation that was successfully tested for the ability to protect humans against *P. aeruginosa* infection[14]. Three fold increase in antigen specific IgG was reported following immunization of CF patients with an OprF-OprI fusion protein[12]. Very high IgG titers were induced in adult mice against OprF, OprI and flagellin following immunization with OprF epitope 8 (amino acid residues 311-341)-OprI-flagellins[15]. As an adjuvant the recombinant flagellin potentially affected the vaccine efficacy[16]. Despite the attractiveness of mucosal vaccination, mucosally administered antigens are frequently not immunogenic. The *P. aeruginosa* OprF protein, a major outer membrane protein that is surface exposed and antigenically conserved in various strains of *P. aeruginosa*, is a promising antigen for a vaccine[17]. It is assumed that the most active epitopes of OprF are located in the C-terminal region, because this part of the protein is located on the surface of the bacterial cell[18]. The pure recombinant and synthetic antigens used in modern day vaccines are generally less immunogenic than older style live/attenuated and killed whole organism vaccines. One can improve the quality of vaccine production by incorporating immunomodulators or adjuvants with modified delivery vehicle[19]. It has

Table 1 Primers and linkers used to amplify and fuse outer membrane protein F and B subunit of LT

Name	Sequence (5′-3′)	Restriction site
OprF-F	TTAA *AAGCTT* ATGAAACTGAAGAACACCTTAG	Hind Ⅲ
OprF-R	TATA *CTCGAG* TTACTTGGCTTCRGCTTCT	Xho Ⅰ
Linker-EAAAK-F	ATAT *AAGCTT* GAAGCTGCGGCAAAA ATGAAACTGAAGAAC	Hind Ⅲ
Linker-EAAAK-R	ATAT *CTCGAG* TTACTTGGCTTCGGCTTCTACTTCGGCTTC	Xho Ⅰ
LTB-F	ATAA*GAATTC*ATGGCTCCGCAAAG	EcoR Ⅰ
LTB-R	ATTA*AAGCTT*TTAGTTTTCCATCGAGATG	Hind Ⅲ

Restriction site sequences are shown in italic.

been well established that the B subunit of LT (LTB) and cholera toxin are powerful immunomodulators with strong adjuvant activity[20,21]. The recombinant LTB is safely and commonly used as an adjuvant to stimulate antibody responses to co-administered protein antigens, and its GM1-binding function is essential for both immunogenicity and adjuvanticity[22]. The aim of this study was to produce a recombinant chimeric protein composed of the OprF fused to LTB in order to evaluate the capacity of this fusion protein to induce a specific immunity in mice burn model against *P. aeruginosa*.

MATERIALS AND METHODS

Kits, enzymes and reagents

Plasmid extraction and gel purification kits were from Bioneer (Daejeon, South Korea). The designed primers were synthesized by Bioneer. Nickel-nitrilotriacetic acid (Ni-NTA) agarose was procured from Qiagen (Valencia, United States). Restriction endonucleases were purchased from Cinnagen (Tehran, Iran). T4 DNA ligase (Fermentas, Vilnius, Lithuania), anti-polyhistidine antibodies and anti-mouse IgG conjugated with HRP (RAY Biotech), nitrocellulose membrane (PROTRAN), microtiter plates (Nunc, United States) were used. All other chemical reagents were from Merck (Darmstadt, Germany) or Sigma (Munich, Germany).

Bacterial strains and growth conditions

E. coli BL21 (DE3) (Invitrogen) and *P. aeruginosa* (PAO1) were grown in Luria Bertani (LB) medium incubated on a shaker at 37 ℃ and 150 rpm.

Construction of OprF and LTB-OprF fusion gene

The *oprF* gene (GenBank accession No.: JX040481.1) was amplified from its genomic DNA by PCR using the OprF-F and OprF-R primers (Table 1). Forward primer was designed to contain a Hind Ⅲ site and reverse primers carried an Xho Ⅰ site. The *OprF* gene was amplified by PCR. Cyclic conditions were initiated at 95 ℃ for 5 min followed by 35 cycles of 94 ℃ for 30 s, 58 ℃ for 1 min, 72 ℃ for 90 s, and a final extension at 72 ℃ for 5 min. The amplified fragments were analyzed on 1% agarose gel. The pET28a (+) vector and PCR products were double digested with Hind Ⅲ and Xho Ⅰ and were then purified using the Bioneer Gel extraction kit. The ligation of OprF into pET28a (+) was performed using T4 DNA ligase. A helix-forming peptide linkers EAAAK was introduced between OPRF and LTB proteins. For the gene fusion with OprF and LTB, DNA was amplified using the *P. aeruginosa* chromosome as a template and oligonucleotide pairs Link-EAAAK-F and OprF-R (Table 1) as primers for the LTB-EAAAK-OprF fusion. Forward primer was designed to contain a Hind Ⅲ site and reverse primers carried an Xho Ⅰ site. In order to construct LTB-OprF fusion Gene, the *OprF* gene with a linker was inserted in Hind Ⅲ and Xho Ⅰ sites of pET28a (+) vector containing LTB gene in EcoR Ⅰ and Hind Ⅲ sites[23]. The recombinant DNA plasmids, OprF-pET28a and LTB-OprF-pET28a were transformed into *E. coli* strain BL21(DE3). The expression host was grown for 12 h at 37 ℃ in LB agar containing 70 μg/mL kanamycin.

OprF and LTB-OprF expression and purification

E. coli BL21 cells harboring the OprF-pET28a and LTB-OprF-pET28a constructs were grown at 37 ℃ under constant shaking at 200 rpm overnight in 10 mL of LB medium containing 70 mg/mL Kanamycin. The culture was then used to inoculate 200 mL of LB medium. 1 mmol/L isopropyl b-D-thiogalactoside (IPTG) was added at the optical density of 0.6 at 600 nm to induce expression. The cells were further incubated for 6 h at 37 ℃ followed by centrifugation at 10000 × *g* for 10 min at 4 ℃. The cell pellet resuspended in lysis buffer (100 mmol/L NaH_2PO_4, 10 mmol/L Tris-Cl, 8 M urea) was sonicated at 200 W at 1 min intervals for five times. The cell suspension was centrifuged at 8000 × *g* for 30 min at 4 ℃ to separate the supernatant from cellular debris. Affinity chromatography was employed to purify the protein from the supernatant using Ni^{2+}-NTA agarose under denaturation condition (Qiagen, CA). A stepwise dialysis was carried out to remove the denaturant (8 mol urea). The fractions were analyzed by SDS-PAGE. Bradford method was used for determination of protein concentration[24].

Western blotting

The purified recombinant OprF, OprF-LTB and bovine serum albumin were electrophoresed on a 12% SDS-PAGE gel to analyze the cross-reactivity. The proteins were blotted onto nitrocellulose membranes. The membrane strips were blocked with 5% nonfat dried milk and washed whit phosphate buffered saline (PBS)

(137 mmol/L NaCl, 2.7 mmol/L KCl and 4.3 mmol/L Na_2HPO_4). The membrane was incubated with rabbit anti-6X His tag antibody (Abcam). This was followed by incubation in 1:10000 dilution of horseradish peroxidase-conjugated (HRP) goat anti-rabbit IgG antibody (Sigma). Detection was carried out using HRP staining solution.

Animals husbandry

The animal care protocol was approved by Shahed University. Four-six weeks old (16-22 g) BALB/c mice procured from the Razi Institute, Tehran, Iran were housed in clean standard animal care facility of Shahed University. The research was carried out in compliance with the Animal Welfare Act and regulations related to experiments involving animals.

Immunization of mice

Mice were divided into three groups of five mice each. Ten micrograms of the recombinant protein was injected subcutaneously (sc) per mouse on days 0, 15, 30 and 45. Equal amount of Freund's complete adjuvant was used at the first dose and incomplete adjuvant in the subsequent doses. Negative control was a group of mice injected with 20 μL of PBS each time simultaneously with the test group.

Determination of Anti OprF and Anti OprF-ltb antibody titers by ELISA

Five micrograms of the recombinant proteins were coated to the surface of each well of 96-well microtiter plates and incubated overnight at 4 ℃. Sera serial dilutions from 1:250 to 1:100000 were added to each well. HRP conjugated anti-mouse IgG (100 μL) diluted to 1:3000 was added to each well as secondary antibody. Orthophenylenediamine (OPD) was added and the reaction was stopped with H_2SO_4 (2 mol/L) in order to detect the immunoreaction. The plates were analyzed at OD_{492} by ELISA reader.

Thermal injuries/ burned mouse model

The mice burned model was carried out according to Stieritz and Holder[25] with slight modifications. Six-eight-week-old female BALB/c mice (22-25 g; Razi Vaccine and Serum Research Institute, Iran) were anaesthetised intraperitoneally using a mixture of ketamine hydrochloride (100 mg/mL; Alfasan, Woerden-Holand) and xylazine (20 mg/mL; Alfasan, Woerden-Holand) in distilled water. A uniform thermal injury of 120 ℃ was brought about by exposing the depilated area for 5 s to a round brass probe of 28 mm diameter heated to thermal equilibration with boiling tap water[26]. Sterile saline (2 mL *i.p.*) was administered to support fluid balance during recovery. Mice were supervised until full recovery. Control mice were shaved but no thermal injury was performed[26].

Determination of bacterial lethal dose (LD_{50})

The mice were inoculated intraperitoneally (*i.p.*) with *P. aeruginosa*. Two hundred microliters of the bacterial suspensions at 3×10^4 to 3×10^9 CFU/mL concentrations were administered subeschar at the burn site to six groups of five BALB/c mice per group. Mortality rate was recorded for three consecutive post-challenge days. LD_{50} was defined as the volume (CFU/mL) of bacterial load that brought about death in half of the population size.

Animal challenge with P. aeruginosa (PAO1)

The microbial population was precipitated at $3000 \times g$ and was then suspended in 0.2 mL PBS. The immunized and control mice were challenged subeschar at the burn site with 10^4 CFU of *P. aeruginosa (PAO1)*.

Bacteriological examination of spleen and liver

Ten immunized and non-immunized mice were sacrificed after 72 h and spleen and liver were removed under aseptic conditions. The surfaces of the samples were washed thoroughly with sterile PBS to remove non adherent bacteria. Tissues were homogenized, and incubated in 1 mL selenite cysteine broth and were subsequently plated on SS agar plates.

In vivo neutralization assays

Antisera to the OprF and OprF-LTB proteins were raised in a New Zealand White male rabbits (Razi institute, Iran) by injecting 100 μg of OprF and OprF-LTB per animal. Lethal dose (10^4 CFU) of *P. aeruginosa* was mixed with 200 μL of immune rabbit serum (diluted to 1:800 in PBS) and was allowed to stand for 30 min at 37 ℃. Groups of five mice were injected intra-peritoneally with the lethal dose of *P. aeruginosa* to study the neutralization[27]. In order to verify that natural antibodies in rabbit serum do not offer any resistance to *P. aeruginosa in vivo*, groups of mice received mixture of lethal dose of *P. aeruginosa* and normal rabbit serum as control[28]. The animals were monitored for mortality for seven days.

Statistical analysis

The data derived from the experiments carried out in triplicate were expressed as mean ± SD. Student's *t* test was used to calculate the *P* values in order to determine the significance of differences in the experimental groups. *P* values of < 0.05 were considered as significant.

RESULTS

Construction and characterization of OprF and LTB-OprF fusion gene

The OprF and Linker-OprF genes of *P. aeruginosa* (PAO1) were successfully amplified by PCR and the fragments of appropriate size and digestion patterns of amplified genes were observed on 1% agarose gel. The amplified OprF gene was cloned into pET28a (+) and amplified Linker-OprF gene was cloned in frame, at the 3' end of the LTB gene carried by plasmid pET28a (+) and

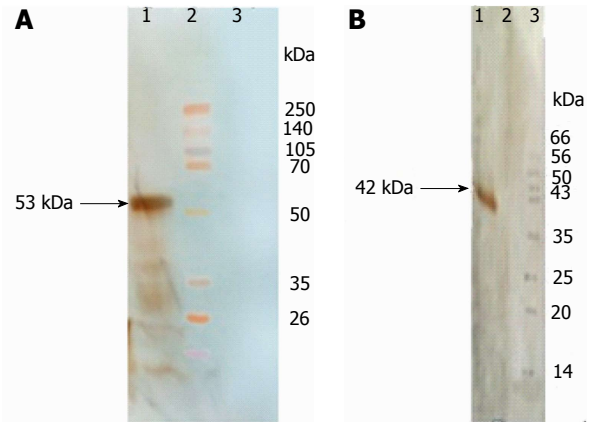

Figure 1 Western blot analysis of recombinant outer membrane protein F (A) and outer membrane protein F-B subunit of LT (B).

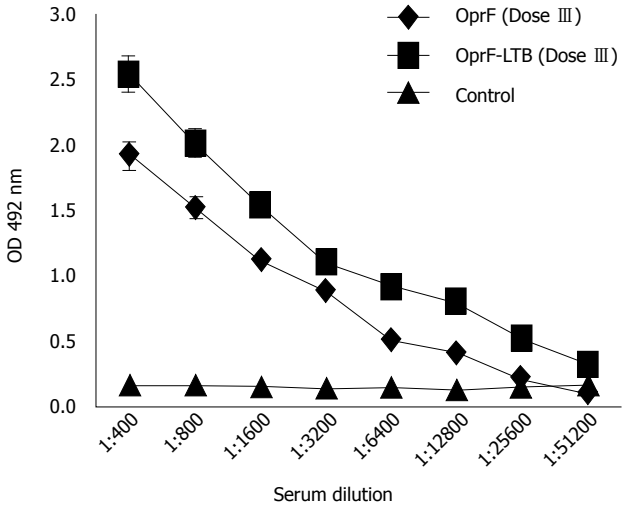

Figure 2 Comparison of IgG titer in mice sera after third dose of immunization with outer membrane protein F and outer membrane protein F- B subunit of LT. OprF: Outer membrane protein F; LTB: B subunit of LT.

confirmed by DNA sequencing. The OprF and OprF-LTB were expressed in *E. coli* BL21 (DE3). The recombinant protein was verified in insoluble pellets by SDS-PAGE. The expression of recombinant proteins was confirmed by reaction with the anti-His-tag antibodies by Western Blotting (Figure 1). Purification of the proteins were carried out under denaturation condition and SDS-PAGE analysis revealed the presence of the approximately 42 kDa (OprF), 53 kDa (OprF-LTB) proteins in the eluted fraction.

Immunogenic property of the recombinant proteins

Animals remained healthy and showed no signs of abnormal behavior after vaccination. Following immunization, mice elicited significant IgG antibodies in sera samples compared to control mice ($P < 0.05$). The antibody titer increased after the second booster, whereas animals received adjuvant and PBS, as a control had no porin-specific antibodies in sera. The combined protein administration had no significant difference with those of the single protein injections (Figure 2).

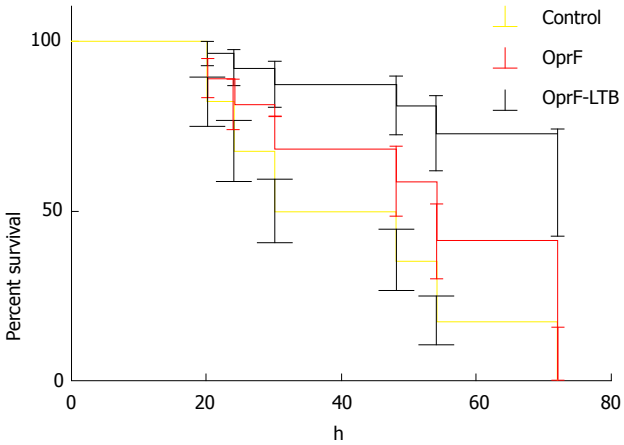

Figure 3 Survival of mice groups challenged with *Pseudomonas aeruginosa*. OprF: Outer membrane protein F; LTB: B subunit of LT.

Animal challenge with P. aeruginosa

LD_{50} was determined as 1×10^3 CFU/mL per mouse *via* injection at the burn site. Percent immunized mice survived is summarized in Figure 3. Control mice died within 24 h of challenge. Mice immunized with OprF-LTB could survive longer than mice immunized with OprF. A significant ($P < 0.01$) survival was observed in mice group immunized with OprF-LTB. The analysis of variance showed significant differences ($P < 0.01$) among the treatments.

Effect of immunization on bacterial uptake in liver and spleen

Mice immunized with OprF-LTB showed high levels of bacteria in outset and reduced gradually to $(1.4 \pm 0.33) \times 10^2$ and $(5.2 \pm 1.11) \times 10^2$ CFU per gram of spleen and liver respectively after nine days. Bacterial loads were detected per gram of spleen and liver of the mice group immunized with OprF $(4.13 \pm 1.06) \times 10^2$ and $(6.1 \pm 0.41) \times 10^3$ CFU respectively. Unimmunized mice exhibited bacterial load of $(6.00 \pm 1.00) \times 10^6$ and $(2.00 \pm 1.00) \times 10^6$ CFU per gram of spleen and liver respectively over the two-week sampling period.

In vivo neutralization assay

The antibody level raised against OprF and OprF-LTB increased in the vaccinated rabbits (Figure 4). Neutralization test was performed to determine if sera from immunized rabbits could protect naive mice against bacterial challenge. As shown in Figure 5, all experimental mice groups were significantly ($P < 0.05$) protected. All mice receiving normal rabbit serum succumbed.

DISCUSSION

P. aeruginosa still remains as a problem in the therapeutic management of nosocomial infections. Today, active

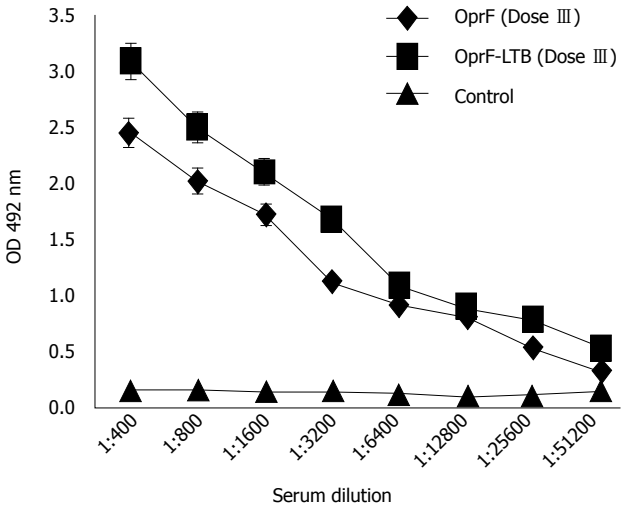

Figure 4 Comparison of IgG titer in rabbit sera after third dose of immunization with outer membrane protein F and outer membrane protein F-B subunit of LT. OprF: Outer membrane protein F; LTB: B subunit of LT.

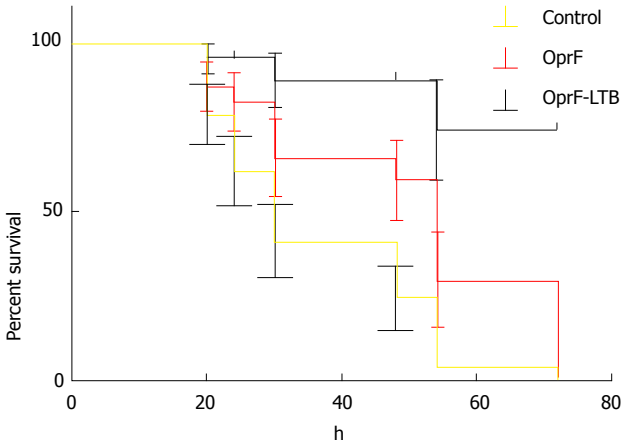

Figure 5 Passive immunization against *Pseudomonas aeruginosa*. OprF: Outer membrane protein F; LTB: B subunit of LT.

and passive immunoprophylaxis against *P. aeruginosa* infections have been widely considered because antibiotic therapy has provided extensive inherent and acquired resistance. As there is a time limitation for burned patients to respond to infectious agents, passive immunization seems to be the best therapeutic pathway for the prevention and treatment of these patients. In this study OprF and fusion genes containing OprF and LTB were cloned and expressed. The results indicate that the LTB adjuvant may enhance the efficacy of the vaccine candidate. A principal effect of LTB interaction with mammalian cells is the stable cross-linking of GM1 at the cell surface, resulting in the uptake of co administered proteins[29], and enhancement of both humoral and cellular immune responses[30-32]. In order to confirm as to whether or not the OprF-LTB and Oprf were immunogenic in mice, serum IgG antibodies were investigated. The serum IgG titers of mice group immunized with OprF-LTB was significantly increased compared to the group immunized with OprF or control group at the 6th week ($P < 0.05$). These results indicate that immunogenicity of LTB in mice. Major structural proteins such as VP2 and VP4 of porcine parvovirus were expressed in *Lactobacillus casei* fused with LTB as a mucosal adjuvant[33,34]. IgG and sIgA levels from mice orally immunized with the fusion proteins were significantly higher than those from mice receiving VP2 or VP4 only without LTB. Our results showed an enhancement the protective efficacy against *P. aeruginosa*. Experimental findings showed significantly higher survival average rate of 75% in burned mice immunized with OprF-LTB than with OprF as well as the control group. Furthermore, the challenge strain isolated from the hepatic and splenic tissues of mice group immunized with OprF-LTB post challenge was significantly lower than those from the group immunized with OprF. However both immunized groups showed significant reduction of bacterial load in spleen and liver compared to the control group. A divided by eight hundred sera dilution from rabbits immunized with OprF and OprF-LTB protected 95% and 75% mice population when mixed with 10 × LD_{50} bacterial load. Antibodies alone can provide relative protective immunity against infection that may partly be related to efficiency of opsonization in deracination of infection[35]. OprF-LTB seems to be used as a vaccine candidate where Pseudomonas infections are potential threat in burn patients.

The results demonstrate that LTB-fused OprF might be a potential candidate as a protective immunogen against *P. aeruginosa*.

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COMMENTS

Background

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic pathogen that infects hospitalized, burned and immunosuppressed patients. Vaccination of high-risk groups may reduce the incidence and spread of infection. The outer membrane protein F (OprF), a major surface exposed outer membrane protein that is antigenically conserved in various strains of *P. aeruginosa*, is a promising immunogen against *P. aeruginosa*. It has been well established that the B subunit of LT (LTB) is a powerful immunomodulator with strong adjuvant activity. In order to confirm as to whether or not the OprF-LTB and Oprf were immunogenic in mice, serum IgG antibodies were investigated.

Research frontiers

A principal effect of LTB interaction with mammalian cells is the stable cross-linking of GM1 at the cell surface, resulting in the uptake of co administered proteins, and enhancement of both humoral and cellular immune responses.

Innovations and breakthroughs

The initial clinical trials on *P. aeruginosa* vaccines established vaccine safety, however the limited effectiveness in preventing subsequent infection clearly evidenced the need for reevaluating correlates of vaccine efficacy. Although a significant humoral response was elicited by lipopolysaccharide (LPS) vaccination, it was not able to prevent subsequent infection brought about by *P. aeruginosa*. However, both LPS vaccines did not meet the approval for routine clinical use

because of their toxicity associated with their lipid A fraction. Outer membrane proteins, LPS and flagellin have been evaluated as vaccine candidates.

Applications

The results demonstrate that LTB-fused OprF might be a potential candidate as a protective immunogen against *P. aeruginosa*. The *oprF* gene may be cloned into plasmid vector, and the plasmid vaccines could be delivered to mice to find its immunogenicity as a DNA vaccine.

Peer-review

This is an interesting study regarding the use of recombinant OprF-LTB protein to prevent *P. aeruginosa* burn infection in mice. The subject is clinically relevant, and the findings of this study are significant.

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Laparoscopic surgery: A qualified systematic review

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Abstract

AIM: To review current applications of the laparoscopic surgery while highlighting the standard procedures across different fields.

METHODS: A comprehensive search was undertaken using the PubMed Advanced Search Builder. A total of 321 articles were found in this search. The following criteria had to be met for the publication to be selected: Review article, randomized controlled trials, or meta-analyses discussing the subject of laparoscopic surgery. In addition, publications were hand-searched in the Cochrane database and the high-impact journals. A total of 82 of the findings were included according to matching the inclusion criteria. Overall, 403 full-text articles were reviewed. Of these, 218 were excluded due to not matching the inclusion criteria.

RESULTS: A total of 185 relevant articles were identified matching the search criteria for an overview of the current literature on the laparoscopic surgery. Articles covered the period from the first laparoscopic application through its tremendous advancement over the last several years. Overall, the biggest advantage of the procedure has been minimizing trauma to the abdominal wall compared with open surgery. In the case of cholecystectomy, fundoplication, and adrenalectomy, the procedure has become the gold standard without being proven as a superior technique over the open surgery in randomized controlled trials. Faster recovery, reduced hospital stay, and a quicker return to normal activities are the most evident advantages of the laparoscopic surgery. Positive outcomes, efficiency, a lower rate of wound infections, and reduction in the perioperative morbidity of minimally invasive procedures have been shown in most indications.

CONCLUSION: Improvements in surgical training and developments in instruments, imaging, and surgical techniques have greatly increased safety and feasibility of the laparoscopic surgical procedures.

Key words: Laparoscopic surgery; Endoscopic surgery; Pancreatic surgery; Rectal resection; Gastrectomy; Gastric cancer; Colon resection; Appendectomy; Esophagectomy; Cholecystectomy

Core tip: This review investigates different applications of the laparoscopic approach on the basis of the current literature and summarizes studies concerning laparoscopic surgery in the different medical fields.

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INTRODUCTION

Laparoscopic surgery has existed since the development of diagnostic laparoscopy in the 1960s. The pioneers of laparoscopic surgery Semm K[1] and Muehe E[1] changed it from a diagnostic to a surgical procedure at the beginning of the 1980s, and it has since become a frequently applied technique for a wide field of indications. The procedure has become the gold standard for many organ systems, with some of the most common being reproductive (particularly gynecological) and digestive (as for cholecystectomy). Significant improvements in surgical training, as well as developments of instruments, imaging, and surgical techniques, have made laparoscopic surgery safe and feasible across different medical fields.

This review summarizes studies on the laparoscopic surgery across different fields and highlights the state of the art, standard procedures in laparoscopic surgery.

MATERIALS AND METHODS

A comprehensive search was undertaken using the PubMed Advanced Search Builder. The exact search strings are listed in Tables 1 and 2. In addition, publications were hand-searched in the Cochrane database and in the high-impact journals such as *Ann Surg*, *Lancet*, *BMJ*, *Brit J Surg*, *World J Gastroenterol*, *Surg Endosc*, *World J Surg*, *Am J Gastroenterol*, *Hernia*, *Am J Surg*, *Langenbecks Arch Surg*, *Arch Surg, Chirurg*, *J Am Coll Surg*, and *Colorectal Dis*. Publications had to meet the following criteria to be selected: review articles, randomized controlled trials, or meta-analyses discussing the subject of laparoscopic surgery (Figure 1). A total of 403 articles were identified and downloaded for full-text review. Of these, 218 articles were excluded for not matching the inclusion criteria and 185 articles were determined to match the search criteria and included in the analysis.

The systematic review was conducted in compliance with the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) guidelines[2]. The study was registered on the PROSPERO international prospective register of systematic reviews (Registration Number: CRD42015019334).

RESULTS

Appendectomy

The systematic reviews and meta-analysis that compared the clinical outcome of laparoscopic *vs* open appendectomy clearly showed an advantage of the laparoscopic procedure[3]. Laparoscopic appendectomy has been proven to be a safe and feasible procedure in the treatment of acute appendicitis and even complicated appendicitis[4,5]. Supporters of the laparoscopic appendectomy maintain that in addition to an improved diagnostic accuracy, the procedure lowers the number of wound infections, results in less pain, faster recovery, and an earlier return to work[6]. However, there seems to be an increase in the operating time for the laparoscopic *vs* open surgery[7]. Earlier reviews reported an increase in incidences of intra-abdominal abscess in the laparoscopic group[8]; later reviews, however, did not find any significant difference between the laparoscopic and open surgery groups[6]. The authors explained the discrepancy in the data as a result of an adapted learning curve, better instruments and imaging quality, and of course more experienced surgeons[6].

Regarding the complication rates of laparoscopic *vs* open appendectomy, there were no significant differences in the overall postoperative complications, pulmonary complications, or postoperative ileus[3]. Considering the postoperative ileus situation after a laparoscopic appendectomy, a significant reduction in the short-term bowel obstruction in pediatric patients and patients with perforated appendicitis were described.

Regarding the long-term bowel obstruction or bowel obstruction requiring surgery, there was a significantly better outcome in the laparoscopic appendectomy group[9]. Patients who underwent the laparoscopic procedure for their appendectomy had shorter hospital stays and were able to "return to normal activity" and "return to work" faster than those who underwent open surgery[3]. The initial higher charges and operating costs in the laparoscopic group were compensated through reduced costs required outside the hospital stay[10].

In the obese patients, the outcome parameters, wound infection rate and overall postoperative complication rate were significantly decreased compared to the open appendectomy group. The operation time did increase, most likely due to technical challenges associated with patients who have a body mass index > 30 kg/m^2. Despite this, the advantages of the laparoscopic approach outweigh the disadvantages for the treatment of appendectomy in obese patients[11].

In pregnant patients, there is a higher risk of fetal loss (although with little evidence) and a slightly increased rate of preterm labor, which was not significant and was without clinical importance[12].

Table 1 Search strategy using publication within the past 5 years

	And	And	And	And	Results	Selected
Laparoscopic surgery	Pancreatic surgery	Whipple	Meta-analysis	Analysis	1	1
Laparoscopic surgery	Rectal resection	Meta-analysis			37	13
Laparoscopic surgery	Gastrectomy	Gastric cancer	Meta-analysis		65	20
Laparoscopic surgery	Liver resection	Liver surgery	Meta-analysis		30	11
Laparoscopic surgery	Colon resection	Meta-analysis			16	6
Laparoscopic appendectomy	Open appendectomy	Meta-analysis			26	13
Laparoscopic surgery	Esophagectomy	Meta-analysis			9	3

Table 2 Search strategy using unrestricted publication dates

	And	And	And	Results	Selected
Laparoscopic surgery	Open cholecystectomy	Meta-analysis		61	15
Laparoscopic surgery	Endoscopic surgery	Groin hernia	Meta-analysis	15	6
Laparoscopic surgery	Endoscopic surgery	Incisional hernia	Meta-analysis	19	6
Laparoscopic surgery	Fundoplication	Meta-analysis		42	9

Newer techniques of reduced ports surgery and single-incision surgery as a less invasive alternative are also performed as a procedure for laparoscopic appendectomy.

The reviews comparing single-incision *vs* conventional laparoscopic appendectomy show a significantly longer operation time with an outcome similar to a conventional laparoscopic procedure with no difference in wound infection rate, conversion to open surgery, reoperation, intra-abdominal collection of fluid, or overall complications[13,14]. Single-incision appendectomy is considered a safe and feasible procedure with comparable clinical outcomes when undertaken by experienced surgeons[15,16]. Results related to better cosmetics and an earlier return to work should be considered with caution due to the small number of studies reporting these two items, along with the short follow-ups used for evaluation of cosmetic results[17]. Critics of the approach have stated that there is no benefit in a single-incision access for appendectomy since clinical outcomes were related to increased operation time, higher frequency of technical failure, and potentially higher costs[18].

Cholecystectomy

Patients who underwent cholecystectomy *via* laparoscopic procedure had no significant difference in morbidity and mortality compared to those who underwent open surgery. In fact, they had faster recovery and shorter hospital stay[19]. Although mini-laparotomy had a similar overall outcome, the authors regard the minimalized open technique as a viable and safe option for healthcare providers without financial resources for laparoscopic equipment and without appropriate, trained surgeons[20]. Later studies showed no difference between laparoscopic and minimally open and conventional open surgery in terms of morbidity and mortality[21].

Laparoscopic cholecystectomy is proven as a safe and feasible technique; concerning cardiac and respiratory complications, it shows superiority over open surgery in elderly patients[22].

Patients with child A or B stadium of liver cirrhosis who underwent laparoscopic cholecystectomy had a similar or even better outcome than those who underwent open surgery. As a result, these patients experienced a lower incidence of postoperative complications[23], such as blood loss and bacterial contamination of ascites - an important cause of in-hospital morbidity and mortality[23]. The postoperative incidence of hepatic insufficiency was not different. These findings have to be regarded with caution, however, because some of the studies had small patient groups with heterogeneous patient populations and criteria[24].

The routine on-table use of intraoperative cholangiography shows no advantage in preventing injuries of the common bile duct or retained stones[25]. There is a significantly longer surgery time with the recommendation not to use routine on-table cholangiography when there are no clinical, radiological or biochemical signs of common bile duct stones[26]. In the management of suspected common bile duct stones, there is a slight advantage concerning complications after sphincterotomy in patients, where the endoscopic sphincterotomy is performed intraoperatively rather than preoperatively[27].

With laparoscopic cholecystectomy, the goal is to minimize trauma to the abdominal wall as much as possible by using the upcoming techniques of reduced port[28-30] or single-port surgery[31] or hybrid NOTES techniques. These techniques have yet to prove their feasibility, safety, and possible superiority over the established 4-port technique of laparoscopic cholecystectomy. In the case of single-incision techniques, the findings varied depending on the number of cases included in the studies. Some of the earlier studies that had been performed when single-incision surgery was newly introduced often lacked evidence and were thus biased.

Some of the studies showed better postoperative pain scores after single-incision procedure *vs* laparoscopic

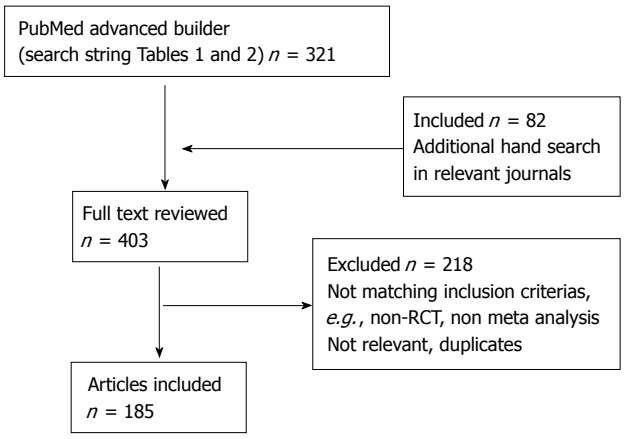

Figure 1 Selection of articles for the review.

procedure[32,33], while others showed no difference[34,35]. Because there was no significant difference in mortality, morbidity, short-term surgical outcome, length of hospital stay, or return to normal activity, many authors consider single-incision cholecystectomy safe and feasible, with a better outcome for patients' cosmetic satisfaction. Others do not recommend single-incision techniques because there is no apparent advantage over conventional laparoscopic cholecystectomy, which has longer operation times and requires additional ports[36]. Hybrid NOTES did not differ in overall complications, surgery-related complications, or postoperative pain, but did have a significantly better outcome in "return to normal activity"[37].

Laparoscopic cholecystectomy is accepted as the gold standard, although studies have shown that mini-laparotomy is similar in outcome, with decreased surgery time and lower cost[19,21]. Single-incision techniques and hybrid NOTES must prove their advantage or similarity in the same way that laparoscopic cholecystectomy has had to do.

Esophageal surgery

Minimally invasive surgery for esophageal cancer is a technically challenging procedure, and the great variability in surgical techniques makes it difficult to interpret the findings and outcomes reported in the current literature. Many studies have a small number of patients, and no randomized controlled trials are available to verify the data[38].

Patients with esophageal cancer who underwent laparoscopy experienced longer surgery time, less blood loss, shorter hospital stay, and reduced overall morbidity[39,40].

One of the difficulties in comparing results across different studies was a lack of definitions regarding postoperative complications or operative techniques. Many studies did not perform or describe a radical lymphadenectomy, and lymph node harvest was lower than the recommended minimum number[41]. Other studies have found a similar lymph node harvest[42] or even more lymph nodes in laparoscopic treatment compared with open surgery[43]. The reported minor complications associated with laparoscopy were similar to open surgery, but the major complications were significantly lower in the laparoscopic group. The lower number of harvested lymph nodes showed a similar percentage of positive lymph nodes, and the lymph node harvest did not impact the survival rate[44], even in the case of the long-term results[45]. A reported adjustment in the surgical techniques (the "Ivor Lewis approach") led to better short-term and long-term outcomes for patients undergoing minimally invasive surgery with an acceptable lymph node harvest and a similar oncological outcome to open surgery[46,47].

Burdall *et al*[48] found significant independent parameters that had an impact on outcome in overall survival. These included the use of neoadjuvant chemotherapy, tumor size (T3 or T4), positivity of lymph nodes, R1-resection, and the surgical approach. Overall, patients who underwent laparoscopic treatment had a significantly better overall survival.

Reflux surgery

The laparoscopic approach has become the gold standard for surgical management of gastroesophageal reflux disease (GERD). Laparoscopic surgery in the treatment of GERD led to reduced hospital stay, faster recovery, faster return to normal activity, and a significant reduction of perioperative morbidity compared with open surgery[49,50]. Laparoscopy has been shown to more effectively treat symptoms, such as reduction of heartburn and regurgitation, and patients reported higher quality of life than achieved with other medical treatments such as proton-pump inhibitor drug alone[51].

The different surgical techniques in the surgical management of GERD are still under discussion. The functional outcome appears similar whether the short gastric vessels are divided or not; therefore, this step in the reflux surgery procedure seems unnecessary and may reduce the operation time if avoided[52-54]. Posterior surgical techniques - the Nissen 360° and Toupet 270° procedures - gave patients a satisfactory control of reflux symptoms. Dysphagia symptoms were more prevalent after the Nissen procedure, while Toupet operations had a higher rate of gas-related symptoms, such as inability to belch and bloating[55]. The Toupet's procedure seems to have superior level 1a evidence than the Nissen procedure. However, these findings should be taken with caution since the study had a Jadad score of 2 with a corresponding possibility of bias[55]. Another evidence-based appraisal for these posterior methods in reflux surgery showed adequate symptom relief after both procedures with a slightly reduced rate of adverse results in the Toupet group[56].

The anterior approach in the treatment of GERD often leads to a longer esophagus acid exposure time, persistent heartburn symptoms and a higher rate of reoperation in the short-term outcomes compared with the posterior approach. However, these symptoms are counterbalanced by the less severe dysphagia

symptoms. For the long-term outcomes, persistence of heartburn and higher use of antacids prevail, with dysphagia scores and gas-related symptoms being similar to those in the posterior group. Hence, there is level 1a support for the usage of posterior techniques in reflux surgery[57].

Robotic surgery used in surgical treatment of GERD is a feasible alternative to laparoscopic surgery. The approach has similar results in terms of symptom relief and surgical outcome. However, lack of obvious advantages with respect to operating time and length of hospital stay and cost do not support the expansion of robotic surgery to other applications in clinics[58].

Gastric surgery

Laparoscopic gastric surgery is a demanding procedure. Results of the surgery depend on the stage of the gastric tumor (early *vs* advanced), whether a partial or total gastrectomy was performed, and presence of gastrointestinal stromal tumors (GIST)[59]. Laparoscopic resections have an overall better short-term outcome with less blood loss, shorter hospital stay, faster recovery of bowel movement with shorter time to first flatus, and fewer serious perioperative complications. However, there was an overall longer time for surgery[60]. Wound complications and surgical site infections were less significant in the laparoscopic group. For severe postoperative complications, such as anastomotic leakage, stenosis, bleeding and postoperative ileus, there was no difference from open surgery[61].

Clearance of harvested lymph nodes is an important factor influencing long-term survival in gastric cancer. Laparoscopic distal gastric resection for early gastric cancer shows fewer or a similar amount of harvested lymph nodes compared with open surgery[60,62,63]. Viñuela *et al*[64] had significantly more lymph nodes harvested in the open surgery group, and the proportion of patients with < 15 lymph nodes was similar to that in the laparoscopic group. The authors were of the opinion that adequate pathological lymph node staging was not compromised by the laparoscopic technique.

Further improvements in the laparoscopic technique show no difference between D2-lymphadenectomy performed for advanced gastric cancer compared to open surgery[65,66]. The surgical approach is not the only parameter that determines the types of or frequency of complications, as well as an acceptable long-term outcome. Other factors such as patient's age, comorbidities (*e.g.,* chronic liver disease, chronic renal failure) and additional organ resection have a significant effect on complications and outcome[67].

In advanced gastric cancer, the total laparoscopic gastrectomy consistently showed the advantages of laparoscopic surgery, having a better short-term outcome than open surgery. Incidence of the short-term complications appears to be correlated with intraoperative blood loss; hence, many studies directed their attention to this parameter. The results in all studies were consistently in favor of the laparoscopic surgery[68].

Concerning the long-term follow-up, the results of laparoscopic surgery were suggested to be similar to open procedures[69]. Current clinical evidence showed that laparoscopic gastric surgery is not inferior to open techniques, even in the treatment of advanced gastric cancer, if the surgeon is experienced[70]. The laparoscopic gastric resection had acceptable results concerning oncological safety[71]. These findings were confirmed in gastric stromal tumors. Laparoscopic surgery was superior to open techniques regarding short-term postoperative outcome without compromising oncological safety and long-term oncological outcome[72]. Furthermore, these long-term results depended mainly on the tumor itself rather than the performed surgical technique[73].

The results of robotic gastric surgery are comparable to laparoscopic surgery. The robotic gastric surgery could be favorably effective considering the increased number of harvested lymph nodes and D2-lymphadenectomy in patients with high body mass index[74]. Data on the long-term outcome and survival are rare, so randomized clinical trials are required[75].

Colorectal surgery

Laparoscopic colorectal surgery is well established as a safe procedure and must be differentiated between the surgical treatment of the benign and malignant disease. The review of current literature shows an ongoing learning curve and an improvement in the management of post-operative care, such as developing fast-track concepts and pathways in "enhanced recovery after surgery" (ERAS). This combination of the surgical procedures (minimally invasive access) reducing major morbidities and standardized postoperative treatment concepts led to a satisfying outcome for patients undergoing colorectal surgery[76]. Colorectal cancer patients who underwent laparoscopic surgery within a multimodal rehabilitation protocol experienced the shortest hospital stay and the lowest morbidity[77].

The general benefits for patients undergoing laparoscopic surgery are the minimal trauma of access, reduced pain, accelerated postoperative return of bowel function, faster return to activity, and better cosmesis. Evidence for potential benefits such as reduced adhesions and a lower rate of incisional hernias was difficult to find[78].

For the ulcerative colitis surgery, the results showed a reduced morbidity in colectomy with a lower mortality in the laparoscopic group compared to open surgery[79].

Surgical treatment of Crohn's disease showed a trend towards lower rate of wound infections, with a lower rate of reoperation in non-disease related complications in the laparoscopic group[80]. In regard to the perioperative morbidity and convalescence, there is evidence that laparoscopic surgery is favored over open surgery for the treatment of Crohn's disease. The data on the lower incidence of small bowel obstruction

and Crohn's recurrence following a minimally invasive surgery was of poor quality[81].

A laparoscopic surgical approach to diverticular disease was associated with longer operation time and reduced postoperative pain. In the elective cases, the reported quality of life score and reduced major morbidity rates were superior to open surgery[82-84]. The decreased incidence of wound infection, the lower need for blood transfusion, and the lower ileus rates led to acceptance of laparoscopic surgery in treatment of elective cases of recurrent diverticulitis[85]. Although laparoscopy minimized the short-term complication rate, a meta-analysis of randomized clinical trials found 42% morbidity in the elective cases, a worrisome finding[86].

The use of hand-assisted devices led to a decrease of conversion rates without difference in the duration of surgery or complication rates. However, all these studies had methodical limitations, along with the additional cost associated with these devices[87]. These findings were less certain in the case of emergency surgery. In generalized peritonitis due to perforated diverticulitis, the approach to drain without resection reduced the incidence of Hartmann's procedure without increasing morbidity and mortality. This finding may lead to a shift in the management of acute perforated diverticulitis in the future[88].

Natural orifice specimen extraction after colon or sigmoid resection is feasible and seems to have an advantage in minimizing trauma to the abdominal wall by avoiding a small incision for specimen extraction. Current literature, however, shows that this technique is not standardized, and no pooled analysis or meta-analysis is practicable, so evidence in the literature is weak[89].

Colon cancer

For colon cancer, the laparoscopic approach is regarded safe and feasible without compromising the oncological outcome. The short-term outcome benefits were lower blood loss, earlier recovery of bowel movement, earlier resumption of oral intake, and reduced hospital stay, as well as lower overall morbidity. The lymph node harvest and results of oncological outcome were similar to open surgery. Thus, laparoscopic surgery was considered superior to open surgery in the short-term outcome of colon cancer[90].

Expert opinion in the literature shows a growing acceptance of laparoscopic surgery for treatment of colorectal cancer, which is further supported by the large randomized clinical trials[91]. Evaluation of the long-term outcomes for non-metastatic colon cancer shows no difference in the occurrence of port site metastasis/wound recurrence, cancer-related mortality, tumor recurrence, or overall mortality[92]. Considering the anatomical circumstances and the oncological outcome, complete mesocolic excision is also recommended in the minimally invasive surgery[93].

For the right hemicolectomy, in regard to the benign and malignant disease, the intraoperative and postoperative results were favorable for the laparoscopic group. There was no difference between the laparoscopic treatment and open surgery in regard to the short-term oncological outcome, including lymph node harvest and length of specimen, and the long-term oncological outcome[94].

In transverse colon tumors, the evidence is not as clear as in other types of colorectal cancer. The benefits of the laparoscopic approach have been demonstrated; however, the data need to be interpreted with caution due to the heterogeneity in the included studies[95].

For long-term survival following laparoscopic colectomy, there was no difference in the overall survival between the laparoscopic group and open surgery. Large randomized controlled trials (excluding transverse colon cancer) demonstrated that laparoscopic surgery was not inferior to open surgery in the long-term oncological outcomes[96]. There was no significant difference in overall mortality, total recurrence rate, or 5-year disease-free and overall survival[97].

Conversion to open surgery seems to have a worse oncological long-term outcome than completion with laparoscopic surgery or starting with an open procedure from the outset. Factors for conversion include higher body mass index, male sex, T3-T4 tumor size, or positive lymph nodal disease[98].

Robotic surgery has short-term outcomes similar to laparoscopic surgery. For blood loss, conversion rate, and time to recover bowel function, there were significant findings in favor of robotic surgery compared with laparoscopy[99].

Rectal surgery

The current data is insufficient to favor a certain technique over another for treatment of rectal prolapse. Laparoscopic rectopexy had fewer postoperative complications and a shorter hospital stay compared with open surgery. The different rectopexy fixation methods did not differ in their outcomes[100]. Compared with a perineal approach (Delorme procedure), the laparoscopic group had higher overall morbidity[101].

Rectal cancer

Rectal cancer laparoscopic surgery has the benefit of minimally invasive access. In the laparoscopic group, blood loss, time to first bowel movement, intake of oral fluids and wound infection were significantly lower after the laparoscopic approach. There were no differences between the laparoscopic approach and the open surgery in terms of complications such as ureter injury, urinary retention, ileus, anastomotic leakage, or an incisional hernia. Oncological outcomes in the short-term and long-term results, such as length of specimen, circumferential resection margin, regional recurrence, port/wound metastasis and distant metastasis, also showed no significant difference[102]. Three-year, 5-year and 10-year

disease-free and overall survival was similar between the two treatments[103].

For early rectal cancer, preoperative staging determines the surgical approach to the rectum. In tumors with good prognostic features and a limited risk of relapse, which can safely be removed while preserving the rectum, the endoscopic mucosal resection or a transanal microsurgery can be performed. If local excision is pathologically difficult or risky, a complete total mesorectal excision (TME) must be done. The laparoscopic approach showed a better short-term outcome with a similar oncological outcome[104].

Contrary to the findings for colon cancer, the clinical outcome was not worse when a laparoscopic procedure was converted to open surgery in rectal cancer. The conversion had no impact on unplanned hospital readmission or redo surgery, nor on 3-year disease-free survival and local recurrence compared with a laparoscopic procedure[105].

Minimally invasive TME can also be performed safely and efficiently after neoadjuvant radiochemotherapy, with a rate similar to open surgery for postoperative complications and a comparable rate of positive circumferential resection margin[106].

Considering postoperative bladder and sexual function in patients undergoing rectal surgery for cancer, there is no difference between the laparoscopic and open surgery group for men in the postoperative ejaculatory and erectile function or bladder function. No difference in the sexual or bladder function between open and laparoscopic surgery was found in women either. Therefore, none of the techniques are superior to the other. Since there is limited data available for these results, they must be viewed with caution[107].

The oncological outcome in laparoscopic surgery was controversial when laparoscopic TME was first introduced. However, the latest results that focus on equivalent oncological outcome prove this topic. The involvement of a circumferential resection margin is equivalent to open TME. Harvest of lymph nodes in the open surgery group was described as slightly higher without having an impact on oncological outcome. In subgroup analysis, there was no difference in R0-resection, distal margin clearance, integrity of the mesorectal fascia, or in local recurrence after 5 years[108,109].

Laparoscopic rectal surgery is a challenging procedure for experienced surgeons. For trainees being supervised by an experienced surgeon, there are similar rates of anastomotic leakage with no difference in the conversion, R0-resection, local recurrence and 30-d mortality compared with experienced surgeons. Furthermore, there were no differences in the cancer-specific survival between the experienced group and the supervised trainees. Therefore, the procedure can be performed by supervised trainees with an adequate learning curve[110].

An upcoming technique is a transanal approach for performing the TME in combination with a laparoscopic approach. In the current literature, the reported TME quality with intact mesorectal fascia is satisfactorily complete, and the number of harvested lymph nodes was $\geqslant$ 12, so the oncological safety parameters seem to be adequate[111].

Robotic procedures in the rectal surgery show the known merits of robotic surgery with a favorable perioperative outcome considering the conversion rate. There were no significant differences with laparoscopic surgery in terms of surgery length, blood loss, recovery of bowel movement, hospital stay, short-term complications and details on the pathological performance such as harvested lymph nodes, resection margin, and circumferential resection margin. These results must be viewed with caution, however, because most of the studies had small sample size or a low level of evidence[112-114]. More high-quality studies are necessary to show the benefit of robotic over laparoscopic surgery and to justify its costs[115]. The long-term benefits of robotic surgery in colon and rectal cancers are still unknown[116].

Liver surgery

Laparoscopic resection of the liver is performed mainly when treating metastasis of colorectal carcinoma or primary hepatocellular carcinoma. Other indications such as cyst resection are rare. The minimally invasive approach is widely applied to patients with limited disease, such as those with solitary findings of 3-4 metastases in the liver. Findings in the liver segments Ⅱ-Ⅳ are better suited for a laparoscopic approach than those in segments Ⅶ, Ⅷ and Ⅳa, which are difficult to reach. Detailed preoperative imaging and an intraoperative ultrasound are helpful[117].

Laparoscopic liver resection has advantages for short-term outcomes such as lower blood loss with a lower rate of transfusion, shorter postoperative hospital stay, and lower rates of positive resection margin and perioperative complications. Patient selection may have influenced some of the observed outcomes[118].

Left lateral resection is described as safe and feasible, and due to lower complication rates and the known advantages of minimally invasive access, some authors claim that it should be used as a standard technique for left resection. At this time, however, there is a lack of randomized controlled trials to support such a statement[119].

Laparoscopic resection with positive short-term results and improved surgical outcome[120] do not compromise oncological outcome or 5-year disease-free and overall survival compared to patients with similar results from open surgery[121,122].

There were fewer postoperative complications in the laparoscopic group concerning short-term outcomes of hepatocellular carcinoma resection, positive resection margin rate, and tumor recurrence, with no significant difference between laparoscopic and open surgery. In short-term results, no tumor recurrence in the site of resection margin and no peritoneal dissemination or

trocar site metastasis were found in the laparoscopic group[123].

The incidence of postoperative ascites and liver failure in the laparoscopic surgery group was lower, benefiting patients with severe liver disease and especially for those with hepatocellular carcinoma[124]. For laparoscopic surgery in the treatment of liver cirrhosis, the advantages for the short-term and long-term outcomes persist[125]. Although laparoscopic liver resection is described as a safe and feasible alternative to open surgery, with favorable outcomes, there is a need for future randomized controlled trials[126] as the current studies do not provide enough support[127].

The laparoscopic approach for radiofrequency ablation is used for hepatocellular carcinoma. Intraoperative laparoscopic ultrasound led to an oncological upstaging in up to 32% in a small case series[128]. In the laparoscopic resection of liver metastases for colorectal carcinoma, these findings do not show any true benefit for the patient, so here, laparoscopy as a diagnostic method appears more as a useful instrument when a peritoneal disease is suspected[129].

Blood loss with a significant reduction of transfusion and the overall complication rate were lower in the laparoscopic group. No difference in the long-term oncological results in 1-, 3- and 5-year survival was found compared with open surgery[130,131]. Some analyses even showed a lower incidence of R1 resection in the laparoscopic group[132].

Resection of liver metastasis can be performed synchronously with colectomy, with a favorable outcome of the short-time results, but there was no difference in the long-term results in regard to the survival or recurrence. The authors consider synchronous resection of liver metastasis to be an option for selected cases, but the included studies lacked standardized inclusion criteria, so the results must be interpreted carefully[133].

In fenestration of congenital hepatic cysts, a favorable short-term outcome of the laparoscopic approach is described without a difference in postoperative complications or cyst recurrence rates, so the minimally invasive surgery is favored in these cases[134].

In living donor liver transplantation, the laparoscopic approach is seen as comparable to the open approach for liver procurement in terms of donor safety. Lower blood loss was shown in the laparoscopic group[135].

Surgery of the adrenal glands

Minimally invasive surgery of the adrenal glands has become the gold standard in resection of benign and malignant disease. Potential laparoscopic techniques are the lateral transperitoneal approach and the posterior retroperitoneal approach. Regarding outcome of these approaches, compared to each other, the posterior retroperitoneal seems to be a comparable[136] or superior[137] approach based on the operation time, blood loss, pain score, hospital stay, and time to return to normal activity[138]. The conversion rate and surgical complication rate are similar[139]. Both techniques have replaced open access to the adrenal gland in tumors $\leqslant$ 8 cm. For the retroperitoneal approach, the findings support a faster convalescence compared with the lateral transperitoneal approach. Since the difference is not significant the authors could not make a recommendation for either approach[140].

Minimally invasive adrenalectomy is the mainstay of operative options for adrenal tumors. Despite lack of evidence for the technique in the randomized controlled trials, it has gained acceptance in the surgical world. Adrenalectomy is performed in hormone-active tumors, lesions > 4 cm, and benign disease with untypical imaging[141].

Studies have shown that the laparoscopic approach can be an acceptable option for the primary neoplasm or adrenal metastasis with no evidence of local invasion[142]. If there is any doubt before the operation that the tumor cannot be removed with an intact capsule, open surgery must be considered[143].

Surgical therapy of primary aldosteronism (Conn's disease), compared with medical treatment, leads to a significantly better decrease in blood pressure, with no difference in the cardiovascular complications in qualitative analyses with heterogeneous protocols. Despite the absence of randomized controlled trials, there is support for unilateral resection of unilateral disease[144].

For subclinical Cushing's disease, unilateral laparoscopic adrenalectomy can resolve the hypercortisolism with low morbidity and can provide significant benefit to blood pressure, glucometabolic control and obesity *vs* a conservative medical treatment. However, these findings lack evidence are from studies that include data that is too low quality for a systematic review, so the authors could not give a definitive recommendation[145].

In the treatment of refractory Cushing's disease, bilateral adrenalectomy plays a crucial role in cases where transsphenoidal surgery of ACTH-producing tumors does not lead to normalization of hypercortisolism. In these cases, laparoscopic bilateral resection is safe and effective[146].

The single-incision technique showed similar benefits to the conventional laparoscopic approach. The studies found no difference in blood loss, length of hospital stay, or time to oral intake, with similar outcomes in cosmetic satisfaction, rate of complication, rate of conversion to open surgery, and need for transfusion[147]; longer duration of surgery, lower pain perception, and slightly faster recovery were also reported[148].

Robotic surgery is used in the retroperitoneal posterior and transabdominal lateral approach. A significantly lower blood loss was found in the robotic group compared with the group who underwent laparoscopic surgery, with no difference in the short-term surgical outcomes or complications[149]. With longer surgery and selection of patients, the widespread robotic techniques will be difficult[150] even with lower conversion rate for large tumors compared with laparoscopic surgery[151]. Training and costs associated with the technique are the

major drawbacks of robotic surgery in the treatment of adrenal glands[152].

Pancreatic surgery

Laparoscopic surgery of the pancreas is a challenging procedure for experienced surgeons. Most of the studies had a small sample size, and patients were highly selected. Laparoscopic left-side resection of pathologies of the distal pancreas or laparoscopic enucleation for benign lesions has been reported as safe and feasible, with morbidity comparable to open surgery[153]. Left-side resection is the most common laparoscopic procedure in pancreas surgery because it is technically easier to perform. The duration of surgery, surgical morbidity, rate of R1-resection and rate of major complications like pancreatic fistula are similar to the open surgery group; hospital stay and blood loss were reduced[154]. Long-term outcomes of the left resection are equivalent[155] even for malignant findings[156].

For laparoscopic necrosectomy, therapeutic drainage is the best option for conservative management. The literature findings were difficult to compare because of differences in the techniques used, as well as in timings of the interventions[154].

Laparoscopic pancreaticoduodenectomy is performed as a laparoscopic-assisted or total laparoscopic procedure[157]. The outcomes were comparable to open surgery in overall morbidity and surgical-related complications; oncological criteria were fulfilled in terms of number of harvested lymph nodes and resection margin. Due to a lack of randomized controlled trials and the small sample sizes of highly selected patients[158], there was a selection bias so the results should be viewed with caution[154].

Robotic surgery was reported only in the case reports or cohort series, making it difficult to compare. Trials were heterogeneous so data could not be analyzed. In the selected patients, results were comparable to laparoscopic surgery[159] with a trend to improve spleen preservation[160].

Surgery of the spleen

Laparoscopic surgery has become standard for splenectomy in elective cases. The laparoscopic approach showed a reduced morbidity, especially in the pulmonary complications after surgery and wound infection compared with open surgery[161].

In large spleens, a hand-assisted device showed an advantage to open surgery in terms of reduced pain and reduced length of hospital stay, with a surgical outcome similar to laparoscopic surgery. Furthermore, compared with laparoscopic splenectomy, the hand-assisted surgery of the spleen had a lower conversion rate to open surgery[162].

Information on the alternative methods of access like NOTES or single-incision laparoscopic splenectomy is rare. These methods are feasible, however, there is a high risk of bias in the early case reports regarding the alternative approaches in highly selected patients[163]. Only single cases on robotic surgery for splenectomy have been reported[164].

Laparoscopic hernia surgery

Groin hernia: Operative and perioperative management of hernia repair represent a wide field of surgical research. Of the tissue repair techniques, the Shouldice procedure is the only technique with recurrence rates that are lower than other open non-mesh techniques. Mesh repair is evidently superior to tissue reconstruction[165]. The Lichtenstein's procedure is the gold standard in open hernia mesh repair, and has minimal recurrence and morbidity.

There are two minimally invasive surgeries available for a groin hernia: A transabdominal preperitoneal repair (TAPP) and a totally extraperitoneal hernia repair (TEP). While the mesh is placed in the same position, the approach through the abdominal cavity (TAPP) or in front of the peritoneum (TEP) is different. Both techniques are described as effective treatments of a groin hernia but with a slight increase in perioperative morbidity in comparison to open mesh techniques[165].

Comparing the two techniques, TEP is associated with a slightly faster postoperative recovery; the TAPP technique has a significantly higher incidence of operative morbidity. For incidence of recurrence, long-term neuralgia, duration of surgery, and length of hospital stay, both laparoscopic approaches seem to be similar[166].

Comparison of the minimally invasive approach with the open mesh repair (Lichtenstein) in unilateral hernias, the TAPP technique had a similar recurrence rate and was associated with greater perioperative morbidity. The TEP technique had a slightly increased recurrence rate and a comparable incidence of perioperative complications. The minimally invasive techniques had a significantly reduced risk of chronic groin pain and groin stiffness[167]. In recurrent hernia repair, neither of the laparoscopic procedures were considered superior to open surgery in terms of recurrence and chronic pain[168].

Use of lightweight mesh in hernia repair reduced the risk of chronic groin pain[169] with no effect on recurrence[170] and showed a reduced risk of stiffness and groin body sensation[169,171].

The type of mesh fixation method in laparoscopic hernia repair led to different outcomes. Fibrin glue showed a reduced risk of chronic groin pain and a higher patient satisfaction[172] without impact on recurrence rate[173]. Use of staplers/tacks had a higher incidence of postoperative and groin pain[174].

In the TEP procedure, mesh fixation was not necessary. In cases where mesh fixation was not used, there was no difference in recurrence, incidence of seroma, or postoperative or chronic pain[175].

In the guidelines for endoscopic hernia repair, the EAES Consensus (European Association of Endoscopic Surgeons), and the International Endohernia Society recommend technical points for a tailored approach to

specific hernias on the basis of evidence[176-178].

Incisional hernia: Laparoscopic surgery for repair of an incisional hernia shows no difference in recurrence or length of hospital stay compared with open surgery. There were significantly fewer wound infections and complications requiring mesh removal[179]. Risk of bowel injury increased, while postoperative pain score showed no significant difference. Long-term results were not available from the current data[180].

A comparison of the data was difficult. There were many studies, reviews and meta-analyses regarding ventral hernia and incisional hernia, although the origin of these hernias was different. Furthermore, the optimal mesh was not found and neither was the optimal mesh fixation[181], which is why long-term results with a high level of evidence are not available[182].

The results on incisional hernia were highly biased due to methodical limitations. It was hard to distinguish whether patient-related factors or technique-related factors, such as type of mesh and fixation, influenced the outcome[183]. Compared with open surgery, there was a longer duration of operation with no difference in the short-term adverse events[184]. The incidence of wound infection was significantly decreased[185]. There was no difference in the overall recurrence. There was a lack of randomized controlled trials with a standardized technique to demonstrate one technique's superiority over the other[186].

DISCUSSION

Laparoscopic surgery has continuously developed over the past years, advancing from an invasive diagnostic tool to an efficient instrument for surgical treatment of benign and malignant disease. Ongoing training, experience, and development in imaging and laparoscopic instruments have facilitated extension of the applications of laparoscopic surgery.

The overall advantage of minimizing trauma to the abdominal wall has been reproducible in many of the laparoscopic procedures compared with open surgery. Faster convalescence, reduced hospital stay, and faster return to normal activity are the most evident advantages of laparoscopic surgery. The outcome, efficiency, decreased incidence of wound infections, and reduced perioperative morbidity of minimally invasive procedures have been shown across different applications, *e.g.,* cholecystectomy, fundoplication, and adrenalectomy. Despite the lack of randomized controlled trials to support laparoscopic applications, these procedures are accepted as the gold standard for surgical intervention. The laparoscopic surgeries are longer than the open ones. However, the duration has decreased over the years through experience and the learning curve. There has been no evidence that open surgery is superior to laparoscopic surgery in terms of oncological short- and long-term outcomes.

Interpretation of the data in meta-analyses or reviews was at times difficult. Different laparoscopic techniques were mixed up under one indication, *e.g.,* in incisional hernia repair. The use of different mesh and types of fixation led to heterogeneity in the compared studies. The possibility of bias as a methodical limitation was high.

Demonstrating the superiority of laparoscopy over other techniques, such as single-incision surgery, NOTES, and robotic surgery was difficult. Cost and effort were the major drawbacks in distribution of these techniques, particularly for the robotic surgery.

Randomized controlled trials in some fields of laparoscopic surgery could not be performed. There is a lack of sufficient evidence, because, for example, sample size calculation to achieve a certain power is too high to conduct such a study in daily surgical routine. There is a need for randomized controlled studies and standardization because many results were interpreted from case reports or cohort series, and were difficult to compare. This is especially needed in technically demanding procedures like laparoscopic gastric, pancreatic and esophagus surgeries.

COMMENTS

Background

Laparoscopic surgery is a technique with a widespread field of applications. In some medical fields, the laparoscopic approach is a standard, *e.g.,* cholecystectomy. Due to the continuous surgical training and the ongoing development of instruments, imaging systems and surgical techniques, highly complex laparoscopic procedures are possible, such as laparoscopic gastrectomy. The primary aim of this review is to show a variety of different laparoscopic surgeries and to assess their benefits and outcomes in benign and malignant diseases.

Research frontiers

This review is a clinical evaluation of laparoscopic surgery and its role in the surgical treatment of benign and malignant disease of different organ systems. Since the first laparoscopic appendectomy and the first laparoscopic cholecystectomy, there has been an overwhelming development of the technique. Therefore, this review shows a complete summary of the most frequent procedures used for digestive diseases and performed with a minimally invasive approach.

Innovations and breakthroughs

No other surgical technique has gone through such advances as laparoscopic surgery over the years, including approaches such as robotic or single-port surgery. This review summarizes the current literature (review articles, meta-analyses, and randomized trials) to give an overview of the laparoscopic procedures.

Applications

Laparoscopic surgery has become a state of the art technique in many fields of surgical treatment, providing better clinical outcomes than open surgery without compromising the oncological results.

Peer-review

The authors have performed a good study. The manuscript is interesting.

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