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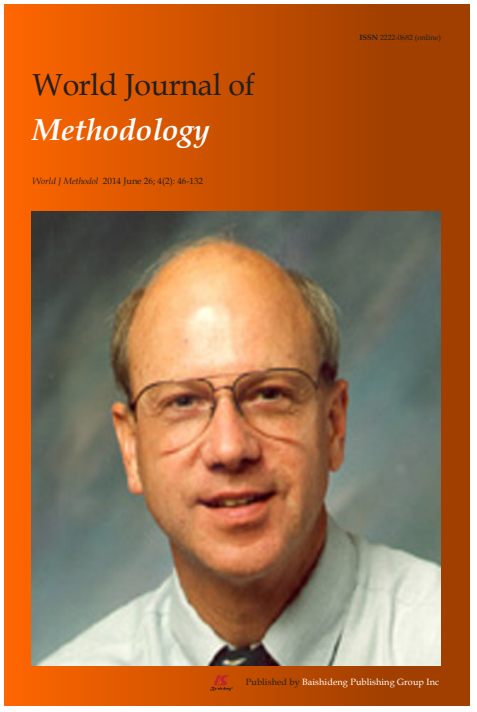

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EDITORIAL

Prospects and advancements in C-reactive protein detection

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Abstract

C-reactive protein (CRP) is one of the earliest proteins that appear in the blood circulation in most systemic inflammatory conditions and this is the reason for its significance, even after identification of many organ specific inflammatory markers which appear relatively late during the course of disease. Earlier methods of CRP detection were based on the classical methods of antigen-antibody interaction through precipitation and agglutination reactions. Later on, CRP based enzymatic assays came into the picture which were further modified by integration of an antigen-antibody detection system with surface plasma spectroscopy. Then came the time for the development of electrochemical biosensors where nanomaterials were used to make a highly sensitive and portable detection system based on silicon nanowire, metal-oxide-semiconductor field-effect transistor/bipolar junction transistor, ZnS nanoparticle, aptamer, field emission transmitter, vertical flow immunoassay *etc*. This editorial attempts to summarize developments in the field of CRP detection, with a special emphasis on biosensor technology. This would help in translating the latest development in CRP detection in the clinical diagnosis of inflammatory conditions at an early onset of the diseases.

Key words: C-reactive protein; Inflammation; Diagnostic methods; Antibody; Biosensors

Core tip: Over time, C-reactive protein (CRP) has emerged as a versatile marker for the detection of systemic inflammatory conditions, providing preliminary information to clinicians for continuing with a more specific diagnostic methodology. Advancements in electroanalytical chemistry and knowledge of nanomaterials have helped modern age researchers to miniaturize detection systems with an enhanced level of specificity and sensitivity of CRP detection. Further research should be directed in this area to devise a better diagnostic platform that can detect the change in CRP level at a very early stage of the onset of inflammatory conditions.

Chandra P, Suman P, Airon H, Mukherjee M, Kumar P. Prospects and advancements in C-reactive protein detection. *World J Methodol* 2014; 4(1): 1-5 Available from: URL: http://www.wjgnet.com/2222-0682/full/v4/i1/1.htm DOI: http://dx.doi.org/10.5662/wjm.v4.i1.1

INTRODUCTION

In humans, there are many acute phase proteins whose level in blood plasma increases or decreases in response to inflammation (acute phase reaction). Some of the acute phase proteins are C-reactive protein (CRP), mannose binding protein, complement factors, serum amyloid A, fibrinogen, retinal binding protein, ceruloplasmin and antithrombin. Amongst them, CRP is the most important, sensitive and systemic marker of inflammation identified in the human body as its level rises rapidly in the blood plasma in response to a large number of foreign bodies, infections, tissue damage, renal and cardiovascular diseases[1]. It is secreted by hepatocytes

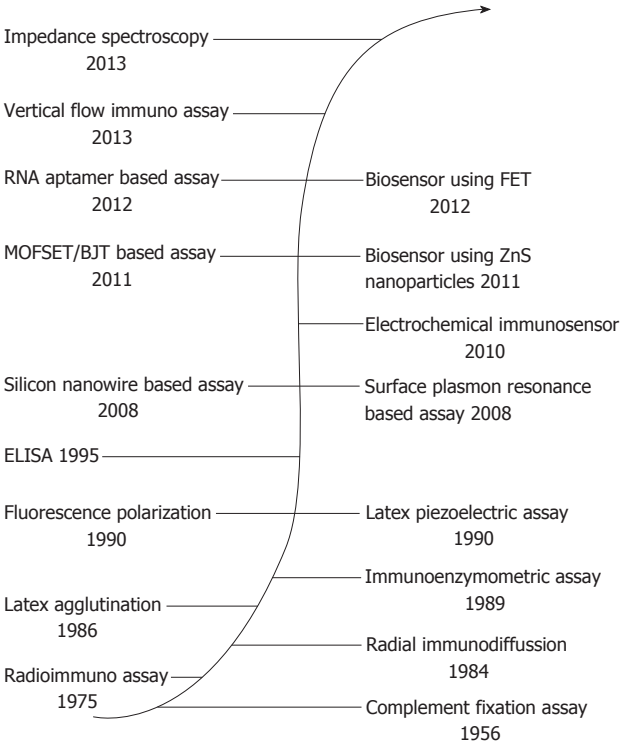

Figure 1 Diagrammatic representation of the advancement in C-reactive protein detection. MOFSET/BJT: Metal-oxide-semiconductor field-effect transistor/bipolar junction transistor; FET: Field effect transistor; ELISA: Enzyme-linked immunosorbent assay.

in response to cytokines, like interleukin 6, interleukin 1, tumor necrosis factor alpha *etc*[2]. CRP (M_r 115,135), a member of the pentraxin family of calcium dependent ligand binding plasma protein, is composed of 5 non-glycosylated polypeptide subunits, each of which is composed of 206 amino acid residues. Polypeptide units associate with each other through non-covalent bonding in an annular configuration forming cyclic pentameric symmetry. The ligand binding site of CRP comprises of loops with two calcium ions. During inflammation, phosphocholine present on necrotic or apoptotic cells binds at the active site of CRP, thereby activating the classical complement pathway essential for opsonization and induction of pro-inflammatory pathophysiological effects. Additionally, it activates the complement pathway but also increases a respiratory burst of neutrophils, encourages expression of adhesion molecules and synthesis of tissue factors. Based on this clinical importance of CRP, attempts have been made in this editorial to summarize the chronological development in the field of CRP detection. The physiological level of CRP in human plasma is 2 mg/L, whereas during inflammatory conditions, its concentration rises significantly in 6-8 h, even reaching up to 300 mg/L in the next 48 h. CRP level in patients with a cardiovascular disorder and/or myocardial infarction at the time of admission to the hospital have been observed to be above the physiological range (more than 3 mg/L)[3]. CRP deposits in the arterial walls during atherogenesis, thereby activating the complement pathway and augmenting the development of several cardiovascular disorders[4]. Abraham *et al*[5] observed a higher level of CRP (14.3 mg/L ± 11.2 mg/L) in patients before dialysis who were susceptible to chronic kidney disorder, renal failure or kidney malfunction. A higher concentration of CRP is also found during late pregnancy. People with obesity and high body mass index also have a higher level of CRP in blood plasma[6]. In a study by Lee *et al*[7], a raised level of high sensitivity CRP (hsCRP) was also correlated with the development of cancer. Hence, CRP is an important marker of clinical conditions like local and systemic inflammation, myocardial diseases, obesity *etc*. The prospect of developing a highly specific and sensitive method of detection of CRP at an early stage of these clinical conditions has been attempted by various research groups. The overall chronological development is elucidated in Figure 1.

Conventional methods of CRP detection rely on precipitation by C-polysaccharide of *Pneumococcus*, tube precipitation, complement fixation, latex agglutination, radioimmunoassay, radial immunodiffusion and fluorescence polarization. Detection of CRP by radial immunodiffusion uses radial immunodiffusion plates made of agarose containing 1% rabbit anti-human CRP. Sera samples are added into the wells punched on them and the diameter of the radial rings measured after a 48 h incubation period. The greater the diameter of the precipitation ring, the higher the CRP concentration in the serum. The time taken for the assay and its semi-quantitative nature are the major limitations of this detection system[8]. As an improvement of the previous technique, the latex agglutination method was developed which employs inert latex particles coated with anti-human CRP antibody. In the presence of CRP in the patient's serum, the agglutination reaction can be seen between anti-human CRP and CRP moieties. Unlike the precipitation reaction, it takes less time but still has the limitation of being semi-quantitative in nature[9]. In 1990, Kurosawa *et al*[10] developed a latex piezoelectric immunoassay using a piezoelectric quartz crystal which acts as the sensing element for the change in viscosity or density in the solution due to aggregation of latex particles. It negated the disadvantages of previous methods of detection of CRP using agglutination through the use of a latex bearing antibody with no film. Earlier piezoelectric assays employed the formation of an antibody coated thin film latex on a crystal by which the oscillating frequency of the crystal reduces. This approach removed the drawbacks of previous methods in terms of labeling reporter molecules and through improving the assay sensitivity. Furthermore, an immunoenzymometric assay for determination of CRP using two antibodies has been developed by Käpyaho *et al*[11]. It is a simple assay consisting of a single immunological reaction between CRP and peroxidase labeled antibody with another antibody attached to the wall of the test tube. The immune complex formed is determined by a colorimetric assay using a peroxidase substrate. The sensitivity of this technique is comparable to the turbidimetric method of CRP detection. However, concerns about enzyme stability, shelf life and time taken for detection raise the question of its practical applications and shelf

life of the diagnostic system[11]. An enzyme-linked immunosorbent assay (ELISA) kit for the detection of CRP (Cell Biolabs Inc., San Diego, CA, United States) has anti-CRP antibody coated onto the microtiter plate that reacts with the CRP antigens. An enzyme linked secondary antibody in the presence of specific substrate gives rise to a colorimetric reaction whose optical density can be measured to estimate the level of CRP. The detection limit of this is up to 0.1 ng/mL but high false positives due to non-specific binding limits the availability of this methodology. Other major disadvantages include the long detection time, lower sensitivity, low stability, cross reactivity with the serum proteins, lack of miniaturization and on-site analysis.

Thus, in recent years, various biosensor based detection systems have been attempted for quick, sensitive and on-site detection of CRP. A biosensor is an analytical device utilizing a biological reaction between receptor and target molecules, converting the biological response into readable and quantifiable signals using transducers[12-15]. Lee *et al*[16] developed a biosensor based on surface plasma resonance spectroscopy which involved measurement of molecular interactions at the gold/silver surface of the sensing element, thereby measuring reflectance of light with respect to the refractive index of the surface of biosensing element that changes when CRP molecular species react at the fabricated unit. This technique uses poly (3-(2-((N-succinimidyl)succinyloxy)ethyl)thiophene) (P3SET) which is a polythiophene with pendant N-hydroxysuccinimide (NHS) ester group as a biolinker between the anti-CRP (bioreceptor) and sensing surface. A self-assembled monolayer (SAM) of P3SET formed on the gold surface and anti-CRP was immobilized covalently. When CRP reacted with sensor, there was a shift in the refractive index of P3SET/anti-CRP due to the formation of P3SET/anti-CRP/CRP on the sensing surface and reflectance was deviated. Hence, the reaction between anti-CRP immobilized on gold surface and CRP can be monitored using surface plasma resonance with a high sensitivity[15].

With advancements in nanotechnology, nanobiosensors have become very popular in recent times. In this regard, Lee *et al*[7] attempted the silicon-nanowire based fabrication process which follows a top-down approach of fabrication using micro-machining technology. In a new study, Yuan *et al*[17] developed a method to adjust sensitivity using a gated lateral bipolar junction transistor (BJT) in the metal-oxide-semiconductor field-effect transistor-BJT hybrid mode which was fabricated using the complementary metal-oxide-semiconductor manufacturing system. Si_3N_4 was immobilized on the layer on gold which was then immobilized on a floating gate using an electron beam evaporator. A die chip consisting of gated lateral BJT was then embedded onto a printed circuit board which was further connected to the vertical collector, base and lateral collector, and emitter. Internal metal layers were also employed to enhance the rate of current flow. Monoclonal anti-CRP antibodies were linked to the gold layer using SAMs of 11-mercaptoundecanoic acid, N-Hydroxysuccinimide and N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride. On reaction with CRP species, capacitance between the liquid and floating gate changes is measured. This change in capacitance has been used to determine the concentration of CRP with high sensitivity and reliability. The advantages of such a system are the small size, ease of manufacturing, low noise, high transconductivity, good selectivity and reproducibility. It has also been claimed that the developed system can be used for other biomarkers by changing the corresponding antibody.

A biosensor integrated with a microfluidic device has been also developed for the detection of CRP. In a report, CRP along with other cardiac marker troponin c has been detected simultaneously using a microfluidic device. The device developed a chip that acted as a microreactor for the simultaneous detection of CRP and troponin c. Antibodies with bioconjugated CdTe and ZnSe were used in the system. These quantum dots release Zn^{2+} and Cd^{2+} ions that are detected by square-wave anodic stripping voltammetry to enable the quantification of the two biomarkers. This electrochemical immunosensor has a detection range of 0.5-200 μg/mL, with a detection limit of 307 attomole in 30 μL for CRP[18]. Another method of detection which uses Zn^{2+} ions for the detection of CRP was established by Cowles *et al*[19] where ZnS nanoparticles were used to transduce the signal *via* fluorescence spectroscopy. In this detection system, mouse anti-CRP coated magnetic microbeads were used. On addition of the serum sample containing CRP, the immune complex binds to these beads to which biotinylated mouse anti-CRP will fix. Neutravidin conjugated with ZnS nanoparticles will attach to this complex and in the presence of Flouzin3, a zinc ion selective fluorescence dye, generate a fluorescence signal. The bioassay possesses a detection limit of 10 pmol which makes it a highly sensitive method to detect CRP. In addition, it is also non-toxic and a less expensive system to fabricate. Another biosensor based on nanomaterial for the detection of CRP level was developed by Qureshi *et al*[20]. The detection system requires the use of specific interaction between CRP and its corresponding RNA aptamer. These CRP specific RNA aptamers are immobilized on carbon nanotubes activated gold interdigitated electrodes of capacitors *via* a physical adsorption. The selective binding of RNA aptamers with CRP is determined by measuring the capacitance after competitive binding between complementary RNA and CRP in pure forms and co-mixtures. It is a label-free method of detection based on affinity separation of target molecules with a limit of detection ranging from 1-8 μmol/L. Although the detection limit is very low, this method has merit in terms of a label-free approach and simple approach for detection of CRP. Kim *et al*[21] recently developed a biosensor using a field effect transistor in which silicon binding protein (SBP) is linked to surface protein A to simplify the tedious method of fabrication of the monolayer. SBP, an artificial protein,

Table 1 Various C-reactive protein detection techniques and their characteristics

No.	Technique employed	Features	Ref.
1	Radial Immunodiffusion	Qualitative analysis in less than 48 h	Harris *et al*[8], 1984
2	Latex agglutination	Time taken less than 24 h; qualitative analysis	Senju *et al*[9], 1986
3	Latex piezoelectric assay	Uses quartz crystal and latex bearing antibody; more sensitive than conventional methods; less time required.	Kurosawa *et al*[10], 1990
4	Immunoenzymometric Immunoassay	Single immunological reaction; sensitive; results comparable to turbidimetric detection	Käpyaho *et al*[11], 1990
5	Surface plasma resonance spectrophotometry	High sensitivity; on-site analysis; SAM usage	Kim *et al*[15], 2008
6	Silicon nanowire based assays	Micro-machining technology; higher detection limit	Lee *et al*[16], 2008
7	MOFSET/BJT based technique	High sensitivity, change in capacitance measurement; reliable; small size; ease of manufacturing; good selectivity; highly reproducible; high trans conductivity	Yuan *et al*[17], 2011
8	Electrochemical Immunosensor	Detection by square wave stripping voltammetry; quantitative analysis of 2 biomarkers; reproducible	Zhou *et al*[18], 2010
9	Nanotechnology using ZnS nanoparticles	Detection by fluorescence spectrophotometry; highly sensitive; non-toxic; low cast system; highly specific	Cowles *et al*[19], 2011
10	RNA aptamer based technology	Uses Carbon nanotube's interdigitated electrodes of capacitors; highly selective	Qureshi *et al*[20], 2012
11	Biosensor using FET	Involves SBP linked in protein A; point of care testing system; on-site analysis	Kim *et al*[21], 2013
12	Vertical flow Immunoassay	One-step assay; time taken 2 min; most rapid; employs gold nanoparticles	Oh *et al*[22], 2013
13	Electrochemical impedance spectroscopy	Most advanced technique; uses gold and diamond spray in fabrication; highly sensitive; reusable without sensitivity being lost; good detection limit	Bryan *et al*[23], 2013

FET: Field emission transmitter; MOFSET/BJT: Metal-oxide-semiconductor field-effect transistor/bipolar junction transistor; SAM: Self-assembled monolayer; SBP: Silicon binding protein.

can bind to the silicon surface with no bi-linker. A fabricated device is treated with hot piranha solution to maximize the affinity of SBP-protein A complex onto the sensing area. The SBP-protein A is then immobilized on the surface of sensing element and dipped into the solution containing anti-CRP. The anti-CRP is coated onto the fabrication unit where CRP forms the immune complex which is transduced in a detectable signal. This is the application of a biosensor point-of-care-testing system with a detection limit comparable to that of ELISA. Oh *et al*[22] has recently developed a one-step biosensor for hsCRP detection using a vertical flow immunoassay. It is composed of a sample pad, flow through films (FTH), conjugate pad and nitrocellulose membranes (onto which anti-hsCRP and secondary antibodies are immobilized below the holes) which are stacked upon one another. Anti-hsCRP conjugated with gold nanoparticles is encapsulated in the conjugate pad. This fabricated system detects hsCRP 0.01-10 μg/mL within 2 min and is the most rapid biosensor to date (Table 1).

Recently, an optimized biosensor for a label-free detection of CRP in a blood serum sample has been developed by Bryan *et al*[23], based on electrochemical impedance spectroscopy using gold electrodes. SAMs of polyethylene glycol (HS-C_{11}-$(EG)_3$-OCH_2-COOH) with the help of ethanol and nitrogen gas are made and dipped into piranha solution. NHS is used to activate the carboxylate group and monoclonal anti-CRP is linked to monolayers covalently. This device detects CRP in blood on the basis of difference in impedance when CRP species reacts with the monoclonal anti-CRP antibody bound to SAM. This system of detection has a very good selectivity and reusability with no loss of apparent sensitivity. This can be considered one of the latest methods of CRP detection where no specific labeling is required *i.e.*, a label free detection system even through the picomolar detection limit.

CONCLUSION

Our understanding of CRP detection systems has come a long way. Over the years, CRP has become a versatile inflammatory marker for the detection of systemic inflammatory conditions. In future, advancements in interdisciplinary approaches will be helpful for the quick, ultrasensitive analysis of these markers. Attempts should also be made to develop new CRP recognition molecules and new material to develop sensing platforms. While developing and implementing these concepts, care should be taken that these systems have promise for CRP analysis in body fluids.

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REVIEW

Off-pump coronary artery bypass grafting: Misperceptions and misconceptions

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Abstract

Coronary artery bypass grafting (CABG) continues to be one of the most commonly performed cardiac surgical procedures worldwide. Conventional CABG performed on cardiopulmonary bypass termed on-pump CABG is regarded as the gold standard. However, on-pump CABG results in several physiologic derangements including but not limited to thrombocytopenia, activation of complement factors, immune suppression, and inflammatory responses leading to organ dysfunction. Furthermore, manipulating an atherosclerotic ascending aorta during cannulation and cross-clamping can predispose to embolization and stroke risk. Recognition of these detrimental effects of on-pump CABG resulted in resurgence of off-pump CABG nearly two decades ago. Off-pump CABG since its resurgence has been a subject of intensive scrutiny and speculation. Despite numerous retrospective nonrandomized studies, prospective randomized trials, and meta-analyses validating the safety and efficacy of off-pump CABG, opponents of the technique have persistently demanded abandonment of off-pump CABG. Several misconceptions and misperceptions are used as an excuse for such demands. This review article examines published scientific evidence to evaluate these misperceptions and misconceptions about off-pump CABG.

Key words: Coronary artery bypass grafting; Cardiopulmonary bypass; Off-pump coronary artery bypass grafting; Surgical myocardial revascularization; Coronary artery surgery

Core tip: There is reluctance to adopt off-pump coronary artery bypass grafting owing to concerns about incomplete revascularization, poor graft patency, and long-term mortality. These concerns are the result of misperceptions and misconceptions rather than reality. This manuscript attempts to tackle these misperceptions and misconceptions.

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INTRODUCTION

Conventional coronary artery bypass grafting (CABG) is characterized by performance of delicate coronary anastomoses on cardiopulmonary bypass (CPB). However, the price of a still and bloodless operative field is ultimately paid by the patients in the form of negative effects of CPB including blood trauma, activation of a series of inflammatory responses, nonpulsatile flow, and possible embolization of air or debris-most particularly embolization of atherosclerotic debris from the aorta[1]. Off-pump CABG was rediscovered with the primary objective of avoiding these deleterious effects of CPB.

Since its resurgence nearly two decades ago off-pump

CABG has been extensively investigated and scrutinized. It has been compared with the gold standard on-pump CABG in numerous randomized controlled trials[2-10] as well as large retrospective observational studies[11-15]. Majority of the published evidence comparing on-pump and off-pump CABG has shown comparable outcomes for these two techniques. However, inability of small, prospective, randomized controlled trials that have lacked sufficient sample size to demonstrate differences in early and long-term outcomes coupled with misperceptions and misconceptions about incomplete revascularization, reduced long-term graft patency and increased need for repeat revascularization resulting in inferior long-term survival have prompted opponents of off-pump CABG to demand abandonment of this technique. Those who question the feasibility and utility of off-pump CABG completely ignore the fact that larger observational studies that are better powered to statistically compare outcomes have shown more favorable in-hospital outcomes and equivalent long-term outcomes with off-pump and on-ump CABG[11-16].

In the current era increasing number of patients with high-risk profile are being referred for CABG. The benefits of off-pump CABG are apparent for patients at high risk for complications associated with CPB and aortic manipulation. Recent studies have demonstrated improved outcomes in higher-risk patients undergoing off-pump CABG[6,17-19]. In view of changing patient profile it will be prudent to acknowledge that off-pump CABG is a valuable technique in the armamentarium of cardiac surgeons and is here to stay. Misleading the cardiac surgical community by using misperceptions and misconceptions and in the process denying patients, particularly those at high-risk for complications due to use of CPB, the opportunity to have safe and effective myocardial revascularization off-pump is not a wise move. This review article examines published scientific evidence to evaluate these misperceptions and misconceptions about off-pump CABG and attempts to allay unnecessary apprehension about the safety and efficacy of off-pump CABG.

Completeness of revascularization

The criticism regarding completeness of revascularization is no longer valid in the current era as technology to safely perform multivessel off-pump CABG has improved tremendously over the past decade. Grafting of vessels on the lateral and inferior aspects is no longer impossible. In fact, the majority of evidence from randomized trials suggests at least equivalent completeness of revascularization[2-7,9,10] (Table 1). Furthermore, it is equally important to understand that completeness of revascularization and number of grafts should not be used synonymously. A more logical way to address the issue of completeness of revascularization is to use the index of completeness of revascularization [number of grafts performed divided by the number of grafts needed (number of graftable vessels with angiographically significant stenoses)][20].

It is important to emphasize that the frequency of complete revascularization reported by various studies comparing off-pump and on-pump CABG is always influenced by relative experience with each technique of the reporting center(s) and surgeon(s). For example, centers where on-pump CABG is used for most cases, and off-pump CABG is used for only a few cases the rates of complete revascularization in the late *vs* early off-pump experience will remain the same highlighting the importance of learning curve as well as case load. Such centers can also have an impact on the final completeness of revascularization achieved by multicentre randomized trials. This fact is exemplified by the Veterans Affairs (VA) Randomized On/Off Bypass (ROOBY) trial[21]. Every year, approximately 4000 isolated CABG procedures are performed in the VA system at 42 cardiac surgery facilities[22]. During the recruitment period of the ROOBY trial only 7 of the 42 centers qualified as high-volume off-pump CABG centers performing at least 50 off-pump cases per year[23]. Since the recognized learning curve for off-pump CABG is between 50 and 75 cases[24] it is not surprising that the ROOBY trial reports incomplete revascularization with off-pump CABG.

Several additional caveats exist regarding reporting of incompleteness of revascularization with off-pump CABG. First, none of the trials or studies reporting incomplete revascularization provides an explanation for failure to completely revascularize the off-pump CABG cohort. Second, from these trials, it is difficult to determine the significance of the ungrafted territory for a

Table 1 Comparison of number of grafts performed and completeness of revacsularization off-pump and on-pump in randomized controlled trials

Ref.	No. of grafts off-pump CABG	No. of grafts on-pump CABG	*P*	Completeness of revascularization
Coronary trial, Lamy *et al*[2] 2012	3.0	3.2	< 0.001	Yes[1]
GOPCABE trial, Diegeler *et al*[3] 2013	2.7	2.8	< 0.001	No
ROOBY trial, Shroyer *et al*[4] 2009	2.9	3.0	0.002	No
DOORS trial, Houlind *et al*[5] 2012	2.9	3.1	0.007	Yes
On-off study, Lemma *et al*[6] 2012	3.0	3.3	0.001	Yes
The Best Bypass Surgery trial, Møller *et al*[7] 2010	3.2	3.3	0.11	Yes
SMART trial, Puskas *et al*[9] 2003	3.39	3.4	NS	Yes
BHACAS trial, Angelini *et al*[10] 2002	2.23	2.31	NS	Yes

[1]Rate of incomplete revascularization (as assessed by the surgeon at the time of surgery) was higher, though the *P* value for the difference was only marginally significant (11.8% *vs* 10.0%, *P* = 0.05). NS: Not significant; BHACAS: Beating Heart Against Cardioplegic Arrest Studies; GOPCABE: German Off-Pump Coronary Artery Bypass Grafting in Elderly Patients; ROOBY: Randomized On/Off Bypass; DOORS: Danish On-pump *vs* Off-pump Randomization Study; SMART: Surgical Management of Arterial Revascularization Therapies; CABG: Coronary artery bypass grafting.

number of reasons. Foremost, it is impossible to determine myocardial viability in the territory left ungrafted, because myocardial viability studies were not used in these trials; thus, the significance of a reduced number of grafts in the off-pump CABG cohort is impossible to predict. Likewise, none of the trials used a myocardium at risk score, which is a potentially valuable tool to aid in determining the true significance of the non revascularized territory because there is a recognized hierarchy of effect, depending on which vessels are left ungrafted and how much myocardium is at risk[25]. Synnergren *et al*[26] examined the effect of incomplete revascularization over a 5-year period in a nonrandomized cohort of 9408 patients. Leaving 1 diseased vascular segment without a bypass graft resulted in no increased risk of death. However, leaving 2 vascular segments ungrafted significantly increased the risk for mortality ($P = 0.01$). Finally, it is important to mention that majority of the trials reporting incomplete revascularization with off-pump CABG report similar early mortality and morbidity rates for the two cohorts[2-5].

Graft patency

Graft failure is one of the major determinants of clinical prognosis after CABG. There has been considerable concern among surgeons and cardiologists that the greater technical difficulty of off-pump coronary revascularization might translate into less precise anastomoses and subsequently diminished graft patency[27]. With conventional on-pump CABG, the 15-year patency rate is > 97%. This is the gold standard that any new revascularization method must compete against[28]. A steep learning curve, distractions caused by cardiac motion or pulmonary insufflations, and construction of anastomoses on a moving target have been implicated as factors responsible for inferior graft patency after off-pump CABG[28].

Interestingly, all concerns about suboptimal graft patency over the years have been predominantly attributed to 2 randomized controlled trials[4,29]. Shroyer *et al*[4] demonstrated that the patency rate of the off-pump arm was lower than that of the on-pump arm on 12-mo angiography, and the 1-year composite adverse outcome rate (death from any cause, nonfatal myocardial infarction, and any reintervention procedure) was higher for off-pump than for on-pump CABG. Such findings do not come as a surprise since the 53 participating surgeons enrolled on average only eight patients per year during the study period and had unacceptably high conversion rates to on-pump surgery (12%) and incomplete revascularization (18%). Moreover, in 60% of the cases a resident was the primary surgeon again raising concerns about the relative inexperience translating into poor graft patency. Another unrecognized confounder that contributed to poor graft patency in the ROOBY trial[4] was the concomitant use of endoscopic vein harvesting (EVH) in 1471 patients (on-pump = 907 and off-pump = 564). The incidence of a patient having 1 or more occluded saphenous vein grafts on follow-up angiography was 41.3% in the EVH group, compared with 28.0% in the open vein harvesting (OVH) group ($P < 0.0001$). Overall saphenous vein graft patency in the EVH group was 74.5%, which was significantly worse than the 85.2% rate in the OVH group ($P < 0.0001$)[30]. Since ROOBY trial was recruiting at a time when EVH was not being widely practiced the poor vein graft patency secondary to EVH can be attributed to learning curve and relative inexperience of the vein harvesters. Poor conduit quality, a consequence of the learning curve for EVH, has been shown to be a predictor of early graft failure, blunted positive remodeling, and greater negative remodeling[31].

The other frequently cited randomized trial supporting the argument of poor graft patency after off-pump CABG is the trial by Khan *et al*[29] reporting decreased patency at 3 mo in the off-pump group. However, closer analysis of this reveals that limited experience of the operating surgeons, consisting of only 98 off-pump procedures, which require a different skill set, during the two years before the study (an average of 25 procedures per surgeon per year) coupled with the relatively low dose of intraoperative heparin, the absence of aggressive antiplatelet therapy with clopidogrel postoperatively, and the failure to use new suction devices to optimize exposure were perhaps some of the confounding factors for poor graft patency[32,33].

Long-term survival

The negative impact of incomplete revascularization and lower graft patency on late mortality rates is well-recognized[34]. Takagi *et al*[35] recently published a meta-analysis of 11 randomized trials demonstrating a statistically significant increase in ⩾ 1 year all-cause mortality by a factor of 1.37 with off-pump relative to on-pump CABG (RR = 1.373; 95%CI: 1.043-1.808). It is extremely important to highlight that the sensitivity analysis in this meta-analysis revealed that the ROOBY trial[4] strongly contributed to the pooled estimate. The aforementioned criticisms of this trial provide an explanation for the inferior survival of off-pump cohort. Furthermore, majority of the recently conducted trials reporting 30-d mortality[2,3,5,6] have not yet reported outcomes for long-term follow-up.

It is expected that once longer follow-up data is available for recently conducted randomized trials, that utilized newer technology for stabilization and exposure and had similar index of completeness of revascularization for off-pump and on-pump CABG, this controversy will be resolved.

CONCLUSION

Although there are numerous clinical studies attesting to the benefits of off-pump CABG[36-38], skepticism, fuelled by misperceptions and misconceptions, persists regarding the safety, efficacy, and equivalence of revascularization with off-pump CABG compared with on-pump CABG[39]. It is extremely important to highlight that off-

pump CABG is a technically demanding strategy and central to all the concerns associated with this technique is the issue of learning curve[1]. The learning curve in off-pump surgery can be safely negotiated with appropriate patient selection, individualized grafting strategy, peer-to-peer training of the entire team, and graded clinical experience (preoperative planning, adequate exposure, proximal anastomoses to the aorta, and distal anastomoses initially to anterior wall vessels, followed by inferior wall vessels and then lateral wall vessels)[40].

Contrary to the proponents and opponents of off-pump CABG, the authors' view is that both on-pump and off-pump CABG have their place in the field of myocardial revascularization. Present day cardiac surgeons must adopt off-pump CABG rather than condemn and castigate it. The rational for this view is the changing profile of patients that are being referred for surgical revascularization. At the same time, technical precision, anastomotic quality, and completeness of revascularization should not be compromised in an attempt to avoid the deleterious effects of CPB unless these short-term risks outweigh any potential long-term benefit.

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REVIEW

Impact of HLA-G analysis in prevention, diagnosis and treatment of pathological conditions

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Abstract

Human leukocyte antigen-G (HLA-G) is a non-classical HLA class I molecule that differs from classical HLA class I molecules by low polymorphism and tissue distribution. HLA-G is a tolerogenic molecule with an immune-modulatory and anti-inflammatory function on both innate and adaptative immunity. This peculiar characteristic of HLA-G has led to investigations of its role in pathological conditions in order to define possible uses in diagnosis, prevention and treatment. In recent years, HLA-G has been shown to have an important implication in different inflammatory and autoimmune diseases, pregnancy complications, tumor development and aggressiveness, and susceptibility to viral infections. In fact, HLA-G molecules have been reported to alternate at both genetic and protein level in different disease situations, supporting its crucial role in pathological conditions. Specific pathologies show altered levels of soluble (s)HLA-G and different *HLA-G* gene polymorphisms seem to correlate with disease. This review aims to update scientific knowledge on the contribution of HLA-G in managing pathological conditions.

Key words: Human leukocyte antigen-G; Pathology; Prognosis; Diagnosis; Treatment; Marker

Core tip: Human leukocyte antigen-G (HLA-G) is a tolerogenic molecule. HLA-G has been shown to have important implications in different pathological conditions where it is reported to alternate at both protein and genetic level. The peculiar immunoregulatory function of HLA-G and its dysregulation in different diseases have led to investigation of its role in pathological conditions in order to define possible uses in diagnosis, prevention and treatment. This review aims to update scientific knowledge on the contribution of HLA-G in managing pathological conditions.

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INTRODUCTION

Diagnosis and prevention of diseases is mainly based on the identification of specific biological markers and drug targets. In view of this, the possibility of easy and fast identification of molecules, for example in biological fluids, seems to be even more necessary.

In recent years, different studies have demonstrated that human leukocyte antigen-G (HLA-G), a non-classical class I molecule, could fulfil this necessity[1-3]. In fact, HLA-G expression and levels in biological fluids, cells and tissues in different pathological conditions have been shown. Several authors reported that the level of soluble

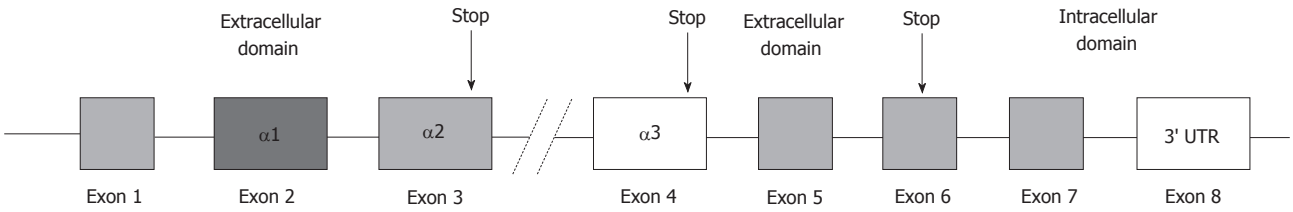

Figure 1 Human leukocyte antigen-G gene. UTR: Untranslated region.

HLA-G and gene polymorphisms correlate with disease outcome and the therapeutic success of treatment[4-6].

HLA-G MOLECULE

HLA-G is a major histocompatibility complex class I antigen encoded by a gene on chromosome 6p21. It differs from classical HLA class I molecules by its restricted tissue distribution and limited polymorphism in the coding region. To date, 50 alleles (IMGT HLA database, August 2013) and 16 proteins are known. The gene structure of HLA-G is homologous to other HLA class I (Ia) genes consisting of 7 introns and 8 exons coding the heavy chain of the molecule. Exon 1 encodes the peptide signal, while exons 2, 3 and 4 encode the extracellular α1, α2 and α3 domains, respectively. Exons 5 and 6 encode the transmembrane and cytoplasmic domains of the heavy chain. Exon 7 is always absent from mature mRNA due to the stop codon in exon 6; exon 8 is not translated (Figure 1). Seven HLA-G isoforms exist due to mRNA alternative splicing and differential association with β2-microglobulin; two of these are found on the cell surface and in biological fluids: Membrane-bound G1 and soluble G5, which lacks the trans-membrane and intracellular domains of membrane-bound G1 (Figure 1)[7]. HLA-G possesses an unpaired cysteine residue at position 42 on an external loop of the peptide binding groove that enables the dimerisation[8,9]. HLA-G monomers are recognized by the inhibitory receptors LILRB1 and LILRB2 and by KIR2DL4[10]. LILR receptors have a greater affinity for the dimeric form that increases the signaling transduction, especially in natural killer (NK) cells[11,12]. The interaction of HLA-G molecules with inhibitory receptors induces apoptosis of activated Crohn's disease ($CD8^+$) T cells[11], modulates the activity of NK cells[13,14] and of dendritic cells (DC)[15,16], blocks allo-cytotoxic T lymphocyte response[17] and induces expansion of suppressor T cell populations, such as $CD4^+CD25^+FoxP3^+$ regulatory T (Treg) cells[18,19]. Moreover, HLA-G is expressed at high levels on DC-10 cells, human DCs with tolerogenic activity and an outstanding ability to produce interleukin (IL)-10[16]. Interestingly, the expression of membrane-bound HLA-G1 and that of its receptors is up-regulated by IL-10 on DC-10 and the expression of high levels of membrane-bound HLA-G1, ILT4 and IL-10 by DC-10 is critical to the generation of allergen-specific Tr1 cells by DC-10[16].

The HLA-G production is controlled by several polymorphisms, both in the promoter and in the 3' untranslated region (3' UTR), modifying the affinity of gene targeted sequences for transcriptional or post-transcriptional factors, respectively[20].

Twenty-nine single nucleotide polymorphisms (SNPs) have been identified in the HLA-G promoter region which may be involved in the regulation of HLA-G expression, considering that many of these polymorphisms are within or close to known or putative regulatory elements. The HLA-G 5' upstream regulatory region (URR) is unique among the *HLA* genes[21] and is unresponsive to NF-κB[22] and interferon (IFN)-γ[23] due to the presence of a modified enhancer A and a deleted interferon-stimulated response element (ISRE). A locus control region located -1.2 kb from exon 1 exhibits a binding site for CREB1 factor, which also binds to two additional cAMP response elements at -934 and -770 positions from the ATG. In addition, a binding site ISRE for interferon response factor-1 is located at the -744 base pair (bp) position[24] and is involved in HLA-G transactivation following IFN-β treatment[24]. The HLA-G promoter also contains a heat shock element at the -459/-454 position that binds heat shock factor-1[25] and a progesterone receptor binding site at -37 bp from ATG[26]. Several promoter region polymorphisms coincide with or are close to known or putative regulatory elements and thus may affect the binding of HLA-G regulatory factors[27]. The -725 C > G/T SNP is very close to ISRE, in which the -725 G allele is associated with a significantly higher expression level compared with the others[28]. The polymorphic sites at the 5' URR are frequently in linkage disequilibrium with the polymorphic sites identified at the 3' UTR, some of them influencing alternative splicing and mRNA stability.

A 14 bp insertion/deletion (INS/DEL) polymorphism (rs66554220) in exon 8 involves mRNA stability and expression[29,30]. In particular, the DEL allele stabilizes the mRNA with a consequent higher HLA-G expression[30,31]. The presence of an adenine at position +3187 modifies an AU-rich motif in the HLA-G mRNA and decreases its stability[32]. One SNP C > G at the +3142 bp position (rs1063320) affects the expression of the HLA-G locus by increasing the affinity of this region for the microRNAs (miR)-148a, miR-148b and miR-152, therefore decreasing the mRNA availability by mRNA degradation and translation suppression[33]. The influence of the +3142G allele has been demonstrated by a functional study in which HLA-G high-expressing JEG-3 choriocarcinoma-derived cells have been transfected with miR-148a, decreasing soluble HLA-G levels. The

discordant results obtained by Manaster *et al*[34], who have reported the lack of +3142 C > G effect on the miRNA control of membrane HLA-G expression, prompt further considerations on the relationship between this polymorphism and membrane HLA-G expression. Other SNPs are identified as implicated in miRNA interaction. In particular, +3003, +3010, +3027 and +3035 SNPs are targets for miR-513a-5p, miR-518c*, miR-1262 and miR-92a-1*, miR-92a-2*, miR-661, miR-1224-5p and miR-433 miRNAs[35]. The miR-2110, miR-93, miR-508-5p, miR-331-5p, miR-616, miR-513b, and miR-589* miRNAs target the 14bp INS/DEL fragment region and miR-148a, miR-19a*, miR-152, mir-148b, and miR-218-2 target the +3142 C/G polymorphism.

HLA-G is a stress-inducible gene; heat shock, hypoxia and arsenite increase different HLA-G alternative transcripts[25,36,37]. The indoleamine 2,3-dioxygenase, an enzyme which metabolizes tryptophan, induces HLA-G expression during monocyte differentiation into DCs[38]. The anti-inflammatory and immunosuppressive IL-10 has been correlated with concomitant HLA-G expression[30,39]. Transactivation of HLA-G transcription has also been demonstrated by leukemia inhibitory factor[40], progesterone[26] and methotrexate[41] cell exposure. Furthermore, IFN-α, -β and -γ enhance HLA-G cell-surface expression by tumors or monocytes[42,43]. HLA-G expression could be acquired by trogocytosis, where a "donor" cell that expresses membrane HLA-G exchanges membrane parts containing HLA-G with a "recipient" cell that is not expressing HLA-G molecules. In this particular situation, "recipient" cells will acquire and make use of membrane HLA-G molecules from a "donor" HLA-G positive cell without the activation of HLA-G gene. Trogocytosis of antigen presenting cell HLA-G1 by T cells in humans makes T cells unresponsive[44]. It has been shown that HLA-G1 can be acquired by NK cells from tumor cells. NK cells that acquire HLA-G1 stop proliferating, are no longer cytotoxic and behave like suppressor cells capable of inhibiting other NK-cell functions[14].

HLA-G's role in immune-tolerance was discovered studying its expression in trophoblast cells at the fetus-maternal interface[45]. The importance of HLA-G production by placental trophoblasts is evident in pre-eclampsia and unexplained recurrent spontaneous abortion (RSA). Several studies have found an aberrant or reduced expression of both HLA-G mRNA and protein in pathological compared with control placentas[46-48], with a possible implication in fetal protection and vascular events.

HLA-G expression has been documented in a few tissues during physiological conditions, such as cornea, thymus, erythroid and endothelial precursors[49-51], and in a variable percentage of serum/plasma samples from healthy subjects[52] where the main producers are activated CD14 positive monocytes[53]. A modified expression of HLA-G molecules has been observed during "no-physiological" conditions, such as viral infection[54-57], cancer[58,59], transplantation[60-64], inflammatory and autoimmune diseases[65,66].

Thus, a growing body of evidence has indicated HLA-G as a suitable key factor in different pathologies. In fact, the immune-modulation by HLA-G may exhibit two distinct effects in pathological conditions: It could be protective in inflammatory and autoimmune diseases[2,65-67], or on the other hand it could be dangerous, for example in tumors or infectious diseases[54-56,58,59]. Based on this evidence, the role of HLA-G in inflammatory and autoimmune diseases has gained considerable clinical interest for the possibility of exploiting it as a molecular biomarker and a therapeutic target.

HLA-G AND PATHOLOGICAL CONDITIONS: PERSPECTIVES IN PREVENTION, DIAGNOSIS AND TREATMENT

Given the immunomodulatory nature of HLA-G molecule, it could be considered a good reference parameter for prevention, diagnosis and treatment in autoimmune and inflammatory diseases.

HLA-G has been analyzed in different pathologies. In this review, we focus on the importance of HLA-G analysis in common and debilitating pathologies characterized by a dysregulation in host immune system in which HLA-G plays a central role.

HLA-G impact in rheumatic disease

Rheumatic disease is a general term used to describe numerous conditions that affect the joints [rheumatoid arthritis (RA)], connective tissues [scleroderma, systemic lupus erythematosus (SLE)] and vessels (vasculitis). Rheumatic diseases are inflammatory and autoimmune diseases, the second most common cause of disability after musculoskeletal injuries. RA (OMIM, #180300) is caused by the immune system attacking synovial cells and treatments include disease modifying anti-rheumatic drugs (DMARDs) and, more recently, biological agents. An important goal of RA therapy has shifted to initiate treatment early and aggressively to achieve remission or low disease activity as quickly as possible. This "treat-to-target" concept has been shown to maximize long-term healthy life[68,69].

Interestingly, RA patients present with an abnormal regulatory network in the immune response, which includes *HLA-G* gene[70]. Serum sHLA-G protein concentration is significantly lower in RA[71] patients than in controls. The decreased sHLA-G concentrations may lead to a chronic activation of inflammatory cells and contribute to the development of the disease. The evaluation of sHLA-G molecules at the specific inflammation site of the synovia reported higher levels of sHLA-G in RA[72] patients. The release of HLA-G in the inflamed synovium may be related to the recruitment of activated HLA-G positive immune cells and the local production by activated synovial fibroblasts[73] that could interact with immune inhibitory receptors and maintain a chronic inflammatory response. These data suggest that there is

a different production of HLA-G molecules on the basis of the local and systemic environments, characterized by different molecular factors and cell types. Interestingly, a recent work confirmed the role of HLA-G molecules in RA. The authors used an intracutaneous treatment of HLA-G monomer or dimer molecules in collagen-induced arthritis model mice. These molecules produced excellent anti-inflammatory effects with a single, local administration[74]. Notably, the dimer exhibited higher immunosuppressive effects than the monomer due to the higher dimer affinity for PIR-B, the mouse homolog of the LILRBs. The HLA-G 14 bp INS/DEL polymorphism has been evaluated as a pharmacogenetic marker of MTX therapy[41]. The authors showed an increase of the 14 bp DEL/DEL genotype in the responder group, characterized by a reduction in disease activity score (DAS28) measured before and after six months of treatment with MTX. In contrast to this study, there are two researches with negative results: (1) 130 RA patients responsive to MTX did not show a significant difference in 14 bp DEL/INS allelic and genotypic distribution (DAS28 < 3.2)[75]; and (2) 186 RA patients, previously untreated with MTX, were prospectively followed up and considered as responders with a DAS28 of up to 2.4 after six months of treatment[76]. No significant association between HLA-G 14 bp INS/DEL and MTX efficacy was observed. Comparing these studies, the discordant results may reflect population differences in gene expression that could influence the power of association studies and lead to different levels of association. In addition, the different doses of MTX and the different cut-off used for RA therapy response definition could affect the results obtained.

Rizzo *et al*[2] evaluated the possible role of HLA-G molecules as biomarkers for RA treatment in a follow-up study. Twenty-three early RA (ERA) patients were analyzed during a 12 mo follow-up disease treatment for sHLA-G levels in plasma samples, mHLA-G and ILT2 expression on peripheral blood CD14 positive cells, and typed HLA-G 14 bp DEL/INS polymorphism. Interestingly, the authors observed that ERA patients with low sHLA-G and membrane HLA-G expression suffered a more severe disease. In fact, sHLA-G levels inversely correlated with DAS28 and ultrasonographic power Doppler scores, used to define the severity and progression of the disease. Interestingly, sHLA-G up-modulation is evident after 3 mo of DMARDs therapy, while a significant reduction in tumor necrosis factor-α levels is evident after 9 mo therapy when a clear amelioration of the disease is evident, with a high specificity for HLA-G detection in EA condition. Moreover, the implication of the HLA-G 14 bp INS/DEL polymorphism is confirmed as the presence of the DEL allele characterizes the patients with a significant improvement in disease status.

SLE (OMIM, #601744) is a systemic autoimmune disease of the connective tissue that can affect any part of the body. Rosado *et al*[77] and Chen *et al*[78] showed higher sHLA-G and IL-10 levels in SLE patients in comparison with healthy controls, while Rizzo *et al*[66] observed lower sHLA-G concentrations in SLE patients. The differences in sHLA-G levels in these two papers could be due to the difference in the analyzed samples (serum or plasma) since it is known that the highest sHLA-G levels are recovered from plasma samples compared with serum collected from the same subjects because of a trapping phenomenon during clot formation that could subtract sHLA-G from the serum[79]. As a proof, Monsiváis-Urenda *et al*[80] evidenced a diminished expression of HLA-G in monocytes and in mature CD83 positive DCs from SLE patients compared with healthy controls. In addition, monocytes from SLE patients showed a decreased induction of HLA-G expression in response to IL-10. Finally, lymphocytes from SLE patients displayed a lower acquisition of HLA-G (by trogocytosis) from autologous monocytes compared to controls. Interestingly, ILT-2 receptor expression is increased on lymphocytes from SLE patients, in particular, in CD3 positive cells, CD19 positive cells, CD56 positive cells and related to IL-10 and anti-DNA antibodies[78]. These results confirm the presence of a HLA-G impaired expression in patients with SLE and a possible role in the pathogenesis. Using a SNP mapping approach, HLA-G gene is reported to be a novel independent locus with SLE interaction[81]. In particular, HLA-G 14 bp INS/DEL polymorphism and HLA-G +3142 C > G SNP were analyzed in a SLE population. SLE patients showed a higher frequency of 14 bp INS allele and 14 bp INS/INS genotype[66]. Moreover, 14 bp INS/INS patients presented the highest disease activity[82]. On the contrary, the evaluation of HLA-G 14 bp INS/DEL polymorphism in a SLE Brazilian population failed to present an association[83], while the +3142 G allele was found to be associated with SLE susceptibility[84]. The +3142 G allele and the +3142 GG genotype frequencies are increased among SLE patients compared with controls[85]. These data support the role of HLA-G molecules in the control of the SLE condition and in particular several results sustain the lower HLA-G expression as a risk factor for SLE development.

HLA-G impact in central nervous system inflammatory diseases

Multiple sclerosis (MS) (OMIM, #126200) is a chronic inflammatory demyelinating and neurodegenerative disease of the central nervous system (CNS) with unknown etiology that is widely considered to be autoimmune in nature[86]. The presence in CSF of detectable sHLA-G levels in relapsing-remitting MS (RRMS) patients and, occasionally, in other inflammatory neurological disorders and non-inflammatory neurological disorders was reported for the first time by Fainardi and coauthors[87]. In addition, sHLA-G levels in CSF are higher in RRMS than in controls and increased, in association with IL-10 values, in RRMS patients without than in those with magnetic resonance imaging (MRI) evidence of disease activity[88]. The importance of sHLA-G level evaluation as a biomarker for MS is confirmed[89]. Of note, in RRMS

patients, CSF concentrations of sHLA-G and IL-10 are positively correlated with inactive MRI disease and CSF IL-10 titers are more elevated in patients with than in those without CSF measurable levels of sHLA-G. These data suggest that CSF sHLA-G levels may modulate MS disease activity acting as anti-inflammatory molecules under the control of IL-10 CSF levels which may enhance sHLA-G production together with the influence due to HLA-G polymorphisms[67]. The existence of high CSF concentrations of sHLA-G in MS patients and their association with clinical and MRI stable disease have been repeatedly confirmed in subsequent investigations in which: (1) An intrathecal production of sHLA-G is more frequent in MS than in inflammatory and non-inflammatory controls and predominated in clinically and MRI inactive compared to clinically and MRI active MS[88]; (2) sHLA-G concentrations reciprocally fluctuate in CSF and serum of MS patients because they are decreased in the serum of clinically stable MS and increased in CSF of MRI inactive MS[65]; (3) CSF levels of HLA-G5 and not those of sHLA-G1 isoforms are increased in MS compared to controls and in MS patients without MRI appearance of disease activity than in those with MRI Gd-enhancing lesions[90]; and (4) CSF values of sHLA-G and antiapoptotic sFas molecules are inversely correlated in MS patients with no evidence of MRI disease activity since CSF concentrations of sFas are lower in MS than in controls and in MRI inactive than in MRI active MS[90]. Interestingly, HLA-G and its inhibitory receptors (ILT-2 and ILT-4) are strongly up-regulated within and around MS lesions where microglia, macrophages and endothelial cells are recognized as the cellular sources[91]. Furthermore, protein HLA-G expression is higher on cultured human MS microglial cells after activation with Th1 proinflammatory cytokines and a novel subpopulation of naturally occurring CD4 positive and CD8 positive Treg cells expressing HLA-G (HLA-Gpos Treg) has been recently described in peripheral blood of MS patients with relapse[92].

Further studies demonstrated that IL-10 contributes to mediating the suppressive activity of CD4 positive HLA-G^{pos} T_{reg}[93] which are highly represented in CSF and inflammatory brain lesions of MS patients as activated central memory T cells capable of migrating from the periphery to intrathecal compartment due to the expression of CCR5[94]. These results strengthen the assumption of an association between HLA-G antigens and MS.

Collectively, these observations provide evidence that HLA-G antigens are likely to be involved in the resolution of MS autoimmunity acting as anti-inflammatory molecules and suggest that HLA-G positive Treg could play a role in the development of a CNS immunosuppressive microenvironment at the sites of inflammation in MS.

HLA-G impact in other inflammatory and autoimmune diseases

HLA-G proves to also be an important biological marker in other pathologies, for example, gastrointestinal and allergic diseases and diabetes.

Inflammatory bowel disease (OMIM, #266600) is the general term for CD and ulcerative colitis (UC), two chronic inflammatory disorders of the intestine which have different clinical, morphological and immunological characteristics.

Torres *et al*[95] studied intestinal samples of UC and CD patients and, by using an immunohistochemistry technique, demonstrated that while UC intestinal cells presented with HLA-G on their surface, CD intestinal biopsies did not. This result combined with high levels of IL-10 found in the lamina propria of the colon of UC patients suggested that HLA-G can regulate the mucosal immune responses in UC. The distribution of the 14 bp INS/DEL polymorphism in UC and CD was investigated by Glas *et al*[96]. They observed an increase of both 14 bp DEL/INS and 14 bp INS/INS genotypes and a consequent decrease of the high producer genotype (14 bp DEL/DEL) in UC subjects in comparison with CD patients. Also, Rizzo *et al*[97] found a different HLA-G expression in UC and CD patients. Non activated peripheral blood mononuclear cells from CD patients spontaneously secrete sHLA-G, while those from UC patients and healthy donors do not. Furthermore, after stimulation with LPS, both cells from CD and healthy subjects show sHLA-G production, while this does not happen in UC patients. This defective production in UC patients seems to be due to an altered secretion of IL-10 in response to inflammation. The different HLA-G expression profiles in UC and CD patients sustain the different etiopathogenesis at the origin of these two diseases. This hypothesis is sustained by the different modulation of HLA-G observed in the two pathologies after therapy[98]. On the basis of this evidence, it is possible to propose sHLA-G and IL-10 levels as diagnostic parameters to facilitate the diagnosis of UC and CD patients.

Asthma (OMIM, #600807) is a chronic disease affecting approximately 300 million people worldwide, with 180000 deaths resulting annually from severe asthma attacks. Asthma is characterized by chronic inflammation in the airway, which consequently narrows more easily in response to a variety of triggers than the airway of a healthy individual. Nicolae *et al*[99] suggested the role of HLA-G as a potential asthma and bronchial hyperresponsiveness (BHR) susceptibility gene. In particular, susceptibility varies depending on whether the mother has asthma or BHR. A G/G genotype at SNP -964G/A in the promoter region was associated with asthma in the offspring of mothers with either asthma or BHR, whereas the A/A genotype was associated with asthma in the offspring of asthma- and BHR-free mothers. Tan *et al*[33] discovered an association between +3142 C > G (rs1063320) and asthma. HLA-G5 is expressed by airway epithelium and is present in the bronchoalveolar lavage fluid from asthmatic patients[100,101]. In addition to the local presence in airways, sHLA-G may also be found in asthmatic subjects outside the lung. The plasma sHLA-G

levels are higher in atopic asthmatic children than in both non-atopic, asthmatic and non-atopic, non-asthmatic children[101]. The 14 bp INS/DEL polymorphism has no impact on plasma sHLA-G levels in the atopic, asthmatic children. Thus, circulating HLA-G may be important as a biomarker and could potentially modulate immune function more broadly, while the local abundance in airways may have a more direct relationship with immune modulation in the mucosa. There is also *in vitro* evidence that the presence of HLA-G may be different in an asthma condition in comparison with physiological status. sHLA-G expression by peripheral blood mononuclear cells is reduced in asthmatic patients[102] while it is increased in asthma induced by isocyanates[103]. This different behavior may represent differences in biological roles in different disease contexts. A loss of HLA-G could reduce immunosuppression and perpetuate inflammation, whereas increased HLA-G in asthma could be an attempt to reassert immunosuppression. Interestingly, HLA-G is differentially expressed during the lung development[104], suggesting a potential role in lung inflammation induction and chronicization.

Allergic rhinitis (AR) (OMIM, #607154) is characterized by a Th2 polarized immune response. sHLA-G molecules are increased in sera of patients with pollen-induced AR studied outside the pollen season[105], during the pollen season[106] and in perennial AR patients[107]. Interestingly, sublingual immunotherapy (SLIT) for AR is able to reduce sHLA-G serum levels in pollen allergic patients[108,109], suggesting a clinical implication as a biomarker of response to SLIT. Interestingly, children with AR have significantly higher levels of sHLA-G molecules than normal controls or children with allergic asthma[110].

HLA-G impact in pathological pregnancies

During human pregnancy, the maternal immune system recognizes and eliminates alloantigens derived from bacteria or virus, but it tolerates genetically different fetal cells, especially extravillous trophoblast cells invading the maternal decidua or entering the spiral arteries. The expression of HLA-G antigens by trophoblasts is of major importance in protecting the fetus from the semiallogeneic response of the mother[111].

The lack of an established immunological tolerance in pregnancy results in an immune response against paternal antigens expressed by the fetus at the placenta, causing severe health problems for both the fetus and the mother. Complications during pregnancy may affect the woman, the fetus, or both. Miscarriage, RSA and pre-eclampsia account for the most frequent pregnancy complications[112] and the dysregulation of the immunological control at the fetal-maternal interface seems to play a role in these pregnancy complications.

Interestingly, there is a reduced expression of both HLA-G mRNA and protein in pathological compared with control placentas[46-48,113]. In pregnant women, there is a peak of sHLA-G levels in plasma samples in the first trimester that is not evidenced in complicated pregnancies[114,115]. In particular, pregnant women with low sHLA-G plasma levels are characterized by a relative risk of 7.12 of developing placental abruption[116].

The lower secretion of HLA-G by maternal immune cells seems to be in part influenced by HLA-G gene polymorphisms, affecting mRNA stability. In particular, the HLA-G 14 bp ins allele decreases mRNA stability[29,117] and protein production[30,39,118-120]. The HLA-G 14 bp INS/DEL polymorphism seems to affect the fetal HLA-G expression as independent studies have reported fetuses carrying the homozygous genotype for the 14 bp INS allele with a significantly increased risk of pre-eclampsia[121-124]. In addition, the 5' URR seems to be implicated in pathological pregnancies[125]. The confirmed role of HLA-G molecules during pregnancy suggests a potential use in clinical practice. Most pregnancy complications are controversial in terms of diagnosis and treatment. As an example, pre-eclampsia can mimic and be confused with many other diseases and none of the signs are specific. The lower levels of sHLA-G detected in maternal plasma and the HLA-G polymorphism association could assist clinicians in an accurate and reliable diagnosis. Moreover, the HLA-G genetic background of the mother could be an a priori sign of an increased risk of complication during pregnancy. These women could be identified and proposed for a stricter follow-up. It is noteworthy that with an appropriate and timely treatment, the success rate is approximately 80%. Therefore, the use of HLA-G as a biological and genetic marker could improve the management of pregnant women. Moreover, the ability to control HLA-G expression in pathological pregnancies and in women with a high risk of pregnancy complications and infertility could be a tool to cure and prevent these conditions with a deep impact, not only for the individual but also for society.

Until now, more than 15000 embryo culture supernatants have been evaluated for sHLA-G expression, with a positive correlation with embryo implantation rate and pregnancy outcome[126]. However further research is needed to investigate HLA-G in assisted reproductive technologies, but recent studies suggest that sHLA-G is a good candidate as a valuable non-invasive embryo marker to improve pregnancy outcome[127]. Three aspects should be taken into consideration: (1) The recognition of a common sHLA-G detection protocol; (2) The necessity to identify a standardized range for positivity; and (3) The comprehension of the factors involved in the differential expression of sHLA-G between equal stage embryos originating from the same woman.

HLA-G impact in tumors

A high frequency of HLA-G surface expression and increased sHLA-G serum levels has been detected in both hematological and solid tumors. HLA-G and sHLA-G expression correlates with a poor clinical outcome in tumor patients, suggesting a role in the immune escape mechanism of tumors. The frequency of HLA-G expression varies between different types of cancer and even between

different studies in the same type of tumor, probably due to the criteria of patient selection and the methodology used. In hematological malignancies, HLA-G expression was documented with a higher frequency in acute myeloid leukemia cases[128], B and T acute lymphoid leukemia and chronic B lymphocyte leukemia[1,129].

HLA-G expression is frequent in choriocarcinoma[45,130,131], breast[132-135], endometrial[136], and ovarian cancers[137]. In digestive tumors, HLA-G expression was described in esophageal squamous cell carcinoma[138], colorectal cancer[139,140], gastric cancer[19], and liver cancer[141]. In relation to increased membrane HLA-G expression in cancer, higher circulating sHLA-G concentrations were described in patients suffering from different types of cancer[142,143].

These data suggest that HLA-G levels might be used as a diagnostic tool to distinguish between malignant and benign tumors and during disease follow-up. Moreover, HLA-G might serve as a possible marker for tumor sensitivity to chemotherapy and as a prognostic marker for advanced disease stage and clinical outcome. HLA-G assay, either in biological fluids or in biopsies, may have a clinical value in diagnosis, staging, or prognosis of cancer, but prospective validation studies should be conducted in order to use it as a biomarker.

Indeed, it would be important to suppress its immune-suppressive expression in cancer. HLA-G blockade in those tumors that express it remains an attractive therapeutic strategy against cancer. Targeting HLA-G-expressing cancer cells would be also important for maximize the efficacy of anticancer therapies. An experimental approach to target HLA-G-expressing cells in a renal cell carcinoma model was the use of HLA-G- derived peptides based on the binding motif to the HLA-A24[144]. HLA-G peptides induced a cytotoxic attack against HLA-G-expressing HLA-A24 tumor cells, suggesting that HLA-G-mediated suppression can be overcome using peptide-derived immunotherapy.

HLA-G impact in viral infections

Host immune defence mechanisms are efficient at eliminating most viral infections. However, some viruses have developed multiple strategies for subverting host immune defences, thus facilitating their spread in the host[145]. Virus-infected cells are protected against attack by NK cells by HLA-G, providing a long-term immunosuppression function. It may be, therefore, that the diminished immune function induced by HLA-G in the host sometimes leads to an advantage for virus progression by helping viruses subvert the host's antiviral defences[146].

Human immunodeficiency virus type 1 (HIV-1) infection is associated with severe and progressive loss of the immune function in infected persons. It is known that HIV-1 protects infected cells from T lymphocytes and NK cell recognition and lyses by classical HLA-A and B down-regulation and non-classical HLA-G molecule up-regulation, respectively. Since the immunoregulatory ability of HLA-G has become known, the involvement of this molecule in the progression of HIV-1 infection has been widely examined. Studies have focused on the expression of HLA-G in monocytes, which are relevant as reservoirs of HIV-1, and in lymphocytes, which are more susceptible to be infected by HIV-1. Monocytes obtained from HIV-1 seropositive patients expressed HLA-G, although only a small proportion of healthy individuals express this molecule[147]. This might be a consequence of highly active antiretroviral therapy (HAART) since a greater proportion of monocytes expressing HLA-G was observed in patients undergoing HAART compared to untreated[148]. T cells obtained from HIV-1 seropositive individuals were found to express HLA-G at a higher proportion[149] and behave as HLA-G$^+$ Treg.

Human cytomegalovirus (HCMV) is a herpes virus causing widespread, persistent human infection in a delicate balance between the progression of the virus and the defences of the host[150]. HCMV has evolved a number of independent strategies to evade the immune system. HLA-G is produced during viral reactivation in macrophages and astrocytoma cells[56] and the percentage of HLA-G-positive monocytes and sHLA-G levels in patients with active HCMV infection were both dramatically higher than in healthy individuals[151]. The up-regulation observed in HLA-G is probably related to a virus-encoded homologue of human IL-10 (cmvIL-10)[151], which prevents NK cell recognition of infected cells.

Evidence also supports a role of HLA-G in human papilloma virus (HPV) infections. In fact, HLA-G may play a role in mediating HPV infection risk[152] and facilitate cervical cancer development[153].

The ability of specific neurotropic viruses to induce the formation of HLA-G in infected neurons, thus conferring protection against NK cells, was demonstrated. For example, herpes simplex virus-1 and Rhabdovirus[154], trigger the expression and up-regulation of membrane and soluble HLA-G molecules in actively infected neurons.

There is also some evidence that HCV and HBV viruses use HLA-G as a strategy to evade the immune response[155-158].

In summary, one of the main mechanisms of virus evasion is the induction of changes in levels of the classical HLA-G proteins. This enables the virus to prevent infected cells from being recognized and attacked by CTL and NK cells. The main challenge would be to block HLA-G up-modulation by viral infection in order to allow the recognition by immune cells.

CONCLUSION

This review has underlined the importance of HLA-G molecules in pathological conditions.

The literature data suggest that HLA-G could be implicated in both risk and disease chronicization where this antigen is characterized by an impaired expression depending on the different disease environment.

In fact, HLA-G proteins seem to be involved in the

Table 1 Summary of the main studies on human leukocyte antigen G and pathological conditions

Topics	HLA-G genetics and polymorphism	Protein	Ref.
Autoimmune and inflammatory pathologies			
Rheumatoid arthritis		Lower plasma sHLA-G levels than in controls	71
		Higher sHLA-G levels in the synovia	73
		Plasma level of sHLA-G correlates with disease activity parameters	2
	Increase in 14 bp DEL/DEL genotype frequency in responsive patients to MTX treatment		41
	Increase in 14 bp DEL allele frequency in patients with improved disease status		2
Systemic lupus erythematosus		Higher level of sHLA-G and IL-10 in plasma that in controls	77,78
	Higher frequency of 14 bp INS allele and 14 bp INS/INS genotype than in controls	Lower concentration of sHLA-G in serum than in controls	66
		Decrease in HLA-G expression in monocytes and DCs	80
	14 bp INS/INS genotype is associated to the highest disease activity		82
	+3142 G allele and +3142 GG genotype are more frequent in SLE and associated to SLE susceptibility		84,85
Multiple sclerosis		sHLA-G levels in MS CSF are higher than in controls	87,88
	sHLA-G levels in MS could be influenced by HLA-G 14 bp and +3142 C < G polymorphisms		67
		sHLA-G level are increased in serum of CFS of MRI inactive MS	65,90
		HLA-G expression in monocytes is lower than in controls	91
		Presence of HLA-Gpos Treg cells in peripherial blood	92
Inflammatory bowel disease Crohn's disease and ulcerative colitis		HLA-G is present on UC intestinal cells but not in CD biopsies	95
	14 bp INS/DEL and 14 bp INS/INS are increased in UC in comparison with CD patients		96
		PBMCs from CD patients secrete spontaneously sHLA-G	97
		Different modulation of HLA-G by therapy in UC and CD	98
Asthma	-964 G < A and +3142 C < G SNPs are associated with asthma	Expression of HLA-G in airway epithelium and airway system	99,100
		sHLA-G plasma levels are higher in atopic asthmatic children	101
		sHLA-G secretion is increased in asthma induced by isocyanates	103
Allergic rhinitis		Higher sHLA-G serum levels than controls	106-110
Pathological pregnancy		Decreased HLA-G expression in placenta than uncomplicated pregnancies	46-48,112,113
Pre-eclampsia	Increased 14 bp INS/INS genotype frequency than uncomplicated pregnancies		120-125
Tumors		Increased HLA-G expression in tumor cells	1,45,128-143
		Higher sHLA-G serum levels than controls	142
Viral infection		Increased HLA-G expression in viral infected cells	145
HIV-1		Increased HLA-G expression in infected monocytes and T cells	147
HCMV		Increased HLA-G expression in infected monocytes	56
		Increased sHLA-G serum levels than controls	151

sHLA-G: Soluble human leukocyte antigen G; IL-10: Interleukine-10; SNPs: Single nucleotide polymorphisms; RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; MS: Multiple sclerosis; IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; AR: Allergic rhinitis; HCMV: Human cytomegalovirus; HIV-1: Human immunodeficiency virus-1; MRI: Magnetic resonance imaging; HLA-G: Human leukocyte antigen G; INS/DEL: Insertion/deletion; CFS: Chronic fatigue syndrome.

regulation of the immune system during autoimmune and allergic conditions, such as gastrointestinal, skin, neurological, rheumatic diseases, in pathological pregnancies and in the immune escape mechanisms during viral infections and tumor transformation. In particular, in these disorders, HLA-G proteins could directly interact with immune cells or control the balance between Th1 and Th2 cytokines. In fact, a disequilibrium in this setting would maintain an inflammatory and immune-deregulated condition.

The comprehension of the specific role and mechanisms of action of HLA-G antigens in the development and progression of inflammatory and autoimmune disorders could justify the use of HLA-G molecules as a marker of inflammation and drug treatment and open up new therapeutic perspectives. Moreover, the definition of the role of HLA-G genetic polymorphisms as risk and pharmacogenetic markers could sustain the clinical relevance of HLA-G typing in the laboratory routine. In particular, the possibility to use simple, non-invasive and standardized tools for HLA-G analysis makes it quickly transferable to the health care system practice. These could help in pathology outcome prediction and support treatment decisions.

As reported in Table 1, there are still contrasting results that need to be taken into consideration. The present challenge is to confirm whether HLA-G molecules have a potential role in prevention and diagnosis of pathological conditions. The perspective to identify pharmacological strategies to control the HLA-G production would represent a concrete possibility to improve the control of inflammation and to guide the therapeutic approach. In fact, the possible use of HLA-G as a therapeutic target is of extreme interest.

The ability to modulate HLA-G molecules on the cell surface and to administer HLA-G molecules[74] seems to be at the basis of these cell therapies, suggesting the importance of further studies on HLA-G role in pathological conditions and the possibility of having a controlled modification of the HLA-G level according to disease status and pregnancy complications.

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MINIREVIEWS

Molecular biomarkers for grass pollen immunotherapy

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Abstract

Grass pollen allergy represents a significant cause of allergic morbidity worldwide. Component-resolved diagnosis biomarkers are increasingly used in allergy practice in order to evaluate the sensitization to grass pollen allergens, allowing the clinician to confirm genuine sensitization to the corresponding allergen plant sources and supporting an accurate prescription of allergy immunotherapy (AIT), an important approach in many regions of the world with great plant biodiversity and/or where pollen seasons may overlap. The search for candidate predictive biomarkers for grass pollen immunotherapy (tolerogenic dendritic cells and regulatory T cells biomarkers, serum blocking antibodies biomarkers, especially functional ones, immune activation and immune tolerance soluble biomarkers and apoptosis biomarkers) opens new opportunities for the early detection of clinical responders for AIT, for the follow-up of these patients and for the development of new allergy vaccines.

Key words: Biomarkers; Molecular allergy; Grass pollen immunotherapy

Core tip: A concomitant approach of the component-resolved diagnosis biomarkers used to guide prescription of grass pollen immunotherapy, particularly important in regions of the world where grass pollen seasons temporal overlap with other types of pollen, together with candidate predictive biomarkers of clinical efficacy for this type of immunotherapy, classified as tolerogenic dendritic cells and regulatory T cells biomarkers, antibodies biomarkers, especially functional ones, immune activation and immune tolerance soluble biomarkers and apoptosis biomarkers, represents a methodological original presentation with an important educational role in the field molecular allergy considered imperative for clinical practice.

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INTRODUCTION

Molecular biomarkers are indicators of biological or pathogenic processes, or reponses to therapeutic interventions, which possess properties that allow their objective (reliable and accurate) measurements in biological samples, and include nucleic acid-based biomarkers, gene expression products, metabolites, polysaccharides and other molecules. These non-imaging markers, with an important role in the development of personalized medicine, can be classified (Table 1) into disease-related and therapy-related biomarkers[1-11]. Such biomarkers can be exploratory, probably valid or valid, according to differences in their scientific proposals, consensus in the medical community and acceptance by regulatory agencies.

The role of biomarkers has become increasingly important in molecular diagnostics and in guiding decisions related to drug development, clinical trials and modern

Table 1 General classification of molecular biomarkers[1-11]

Biomarkers	Definitions, comments
Disease-related genomic and proteomic biomarkers	
Disease risk biomarkers	Biomarkers associated with the risk of a disease
Diagnostic biomarkers	Indicators of the presence of a disease in an individual, including molecular diagnosis, early disease detection and screening biomarkers
Disease staging biomarkers	Biomarkers for assessing disease severity
Disease prognostic biomarkers	Indicators of the likely course/outcome of a disease for an individual; originally defined as markers that indicate the likely natural course of a disease in an untreated individual, also used to define the baseline risk that suggest the likely outcome of a disease independent of treatment
Drug-related biomarkers (provide information about a patient's response to a therapeutic intervention)	
Pharmacogenomic biomarkers	Defining a DNA or RNA characteristic that is indicator of a response to a therapeutic intervention, facilitate the combination of therapeutics with diagnostics through pharmacogenetics (the study of genetic influence on drug response) and pharmacogenomics (the study of how genomic variation influences drug response)
Proof-of-mechanism biomarkers	Assess, in clinical trials, whether a drug has impacted its target
Drug activity biomarkers	Track the effect of a therapeutic intervention in accordance with its mechanism of action
Pharmacodynamic biomarkers	Measure the effect of a drug on the disease and determine the most effective dose for the patient, as efficacy biomarkers
Toxicity biomarkers	Determine the underlying susceptibility of a patient for a particular side effect or group of side effects
Surrogate biomarkers	Intended to substitute a clinical endpoint in clinical trials and expected to predict clinical benefit
Integral biomarkers	Used in clinical trials for eligibility, stratification, or treatment assignment
Integrated biomarkers	Intended to be used in clinical trials for hypothesis generation or testing, without impact on the treatment
Predictive biomarkers	Pretreatment or baseline measurements used to predict the patient response to a particular treatment

personalized therapy. Significant progress has been made in the scientific research of oncology and neurological biomarkers, and also in the field of inflammatory and immunological biomarkers[12-14].

Allergen-driven inflammation is the key pathogenic mechanism in respiratory allergies. Standard treatments, such as receptor agonists (glucocorticosteroids, beta$_2$-agonists), inverse agonists or antagonists (nonsedating H_1 antihistamines, $CysLT_1$ leukotriene receptor antagonists) are used to treat symptoms, without eliminating the cause of allergy. Because conventional pharmacotherapy fails to restore dysregulated immune responses and, in some patients, to totally control clinical manifestations of allergy, there is a need for new treatment strategies. Although therapeutic tools for manipulation of gene expression in allergic diseases has received increased attention in the emerging era of functional genomics[15], only allergy immunotherapy (AIT) that aims to induce immune tolerance to allergens has reached a good level of robustness as an evidence-based therapy and is currently the only treatment with long-lasting clinical effects with the potential to modify the natural course of the disease. For allergic rhinitis and asthma, AIT is effective in reducing symptom scores and medication use, improving quality of life, and inducing favorable changes in specific immunological markers[16]. The diagnosis of respiratory allergy is usually based on skin prick tests and/or the measurement of allergen-specific IgE in serum. Currently, two types of AIT are in clinical practice: subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT). SLIT is a valid non-invasive and better tolerated alternative to SCIT. Special indications of this local type of immunotherapy exist in patients uncontrolled with optimal pharmacotherapy, in whom pharmacotherapy induces undesirable side effects, those who do not want to be on long-term pharmacotherapy or refusing injections[17,18]. SLIT tablets, with the convenience of self-administration, fulfill the requirements from the regulatory agencies that make mandatory pharmaceutical quality and are authorized as drugs available for grass pollen allergy[19]. Intranasal and intrabronchial immunotherapies are not commonly used because of administration-associated local symptoms[16]. New routes for grass AIT are under evaluation in clinical trials (intralymphatic into inguinal lymph node under ultrasound control, epicutaneous *via* patch type epidermal delivery system)[20,21]. Second generation AIT vaccines based upon recombinant allergens (combined with mucoadhesive vector systems in sublingual products) are being developed as an alternative to conventional allergen extracts[22]. A mixture of different wild-type recombinant grass-specific allergen components of Timothy grass, adsorbed onto aluminium hydroxide, was studied as SCIT in grass pollen allergy, some of them being strong candidates for use as therapeutic vaccines[23,24]. Recombinant allergens for AIT aim to overcome the problems of natural extracts as they can be produced in unlimited amounts with exact physicochemical and immunological properties[25].

Currently, molecular diagnostic biomarkers can be used to guide AIT in the frame of component-resolved management of allergic diseases[26]. Identification and validation of biomarkers that are predictive of AIT clinical response are still unmet needs[16]. Recent advances in molecular biotechnology are destined to revolutionize immunotherapy treatments[27].

The major global health problem represented by respiratory allergies is due to their high prevalence, significant influence on quality of life and strong impact on work and school performance, productivity and economic burden. Allergic rhinitis is estimated to affect some 1.4 billion people globally and asthma is estimated to affect 300 million individuals worldwide. Respiratory allergies

affect all age groups and frequently coexist in the same subjects[28-31].

Pollen allergy is a public health threat of pandemic proportions. The most common outdoor allergens responsible for respiratory allergies are the pollen grains of anemophilous plants (wind-pollinated plants), such as of grasses, trees and weeds, each with specific seasons. Exposure to pollen grains depends of the plant type, wild spreading or cultivation, geographic area, altitude, air currents, temperature, precipitation and other weather events. Grass pollen is an important cause of pollinosis with a remarkable clinical impact all over the world. Its frequency differs regionally, but in many parts of the world, grass-induced respiratory allergy is the most common pollen allergy[27,32,33].

In the search for genomic biomarkers, some researchers tried to identify genetic variants associated with pollen sensitization. In studies performed more than a decade ago, susceptibility to grass allergy was associated with an increased frequency of HLA-DQB1*0301 when compared with the control population[34], while by both non-parametric and parametric statistical methods, scientists found significant associations between specific IgE to ryegrass group 1 and 2 allergens with HLA-DR3[35] and specific IgE to ryegrass group 3 allergens with HLA-DR3 and DR5[36]. A recent genome-wide meta-analysis revealed genetic variants associated with grass pollen sensitization in European adults. The HLA variant rs7775228 (6p21.32), which *cis*-regulates HLA-DRB4, was strongly associated with grass sensitization ($p_{grass} = 1.6 \times 10^{-9}$). Single nucleotide polymorphism (SNP) rs2155219, located at 11q13.5, upstream of chromosome 11 open reading frame 30 and downstream of leucine-rich repeat containing 32, was also strongly and consistently associated ($p_{grass} = 9.4 \times 10^{-9}$). The third-strongest association ($p_{grass} = 1.2 \times 10^{-8}$) was for rs17513503 located at the 5q22.1 locus near transmembrane protein 232 and solute carrier family 25, member 46. SNP rs1898671 from thymic stromal lymphopoietin gene showed weak association with grass sensitization ($p_{grass} = 9 \times 10^{-3}$)[37]. In a Japanese study on matrix metalloproteinase 9 gene SNPs and pollen allergy in children, a haplotype associated with -1590T and 668Q revealed a significant association with cedar pollinosis and orchard grass pollinosis (corrected $p = 0.0012$ and $p = 0.0059$, respectively)[38]. Although findings from such studies could enhance the understanding of immunological mechanisms involved in the pathogenesis of pollen allergy, with possible implications for prevention and treatment, additional scientific data are needed to evaluate genetic determinants, not only for IgE sensitization, but also for potential circulating biomarkers.

Currently, component-resolved diagnosis (CRD) biomarkers can be used to evaluate sensitization to grass pollen allergens. In patients with multi-sensitization, sensitization to cross-reactive panallergen biomarkers, specific IgE to profilins and/or polcalcins, may reduce the anticipated response to pollen AIT. In patients with mono-/oligo-sensitization profiles, major species-specific non-glycosylated allergen biomarkers, specific IgE to *Poaceae*- and *Pooideae*-specific molecules, suggest suitability for AIT[26].

A better understanding of the AIT mechanisms of action to induce peripheral tolerance to allergens is useful to identify proper candidate predictive biomarkers for AIT efficacy: biomarkers of tolerogenic dendritic cells (DCs), T cell biomarkers, antibody biomarkers, immune activation and immune tolerance soluble biomarkers, and apoptosis biomarkers[39-44].

Molecular biomarkers for grass pollen immunotherapy are summarized in Table 2.

COMPONENT-RESOLVED DIAGNOSTIC BIOMARKERS

Used to guide prescription of grass pollen immunotherapy

Recognition of disease-causing allergen components involved in pollen allergy, using the specific IgE against recombinant allergen components as molecular biomarkers, is of utmost importance, especially in patients with multiple sensitizations to different pollen types from plants, with total or partial, temporal and spatial overlap of significant airborne pollen concentration periods. This is particularly imperative in patients with a clinical suboptimally informative history, in regions of the world with great anemophilous plant biodiversity and/or areas where unrelated plants have pollination seasons which are at least partially concomitant in some months of the year[45]. Retrospective symptom assessment is not a reliable method as grass pollen symptoms interfere with the recollection of symptoms induced by other pollen[46].

There is general consensus that AIT should be indicated in patients presenting with established clinical relevance for an allergen source. When seasonal symptoms point to grass pollen allergy, *in vivo* and/or *in vitro* testing typically confirm the presence of specific IgE to this type of pollen. In cases of IgE-sensitization to more than one pollen source from grasses, trees or weeds, it is essential to identify the clinically significant pollen types and exclude any source that may appear involved due to cross-reactivity, thus misrecognizing the primary sensitizing source, and compromising the expected immunological responses to AIT[26].

Grasses are universally distributed. Grass pollen grains are produced by wild or cultivated herbaceous plants (Table 3) belonging to *Liliopsida* class, *Poales* order, *Poaceae* family (*Gramineae*).

The most abundant allergenic grass pollen in many temperate regions originates from tall grasses (up to 1.4 m tall), such as *Phleum pratense*, *Dactylis glomerata* and *Arrhenatherum elatius*. Cultivated rye also has a remarkably high pollen production. Allergenic cross-reactivity between the members of the *Pooideae* subfamily grasses of temperate regions (*Lolium perenne, Phleum pratense, Poa pratensis*) is extensive, but it is limited with other tropical or

Table 2 Molecular biomarkers summarized for grass pollen allergy immunotherapy[26,39-44]

Biomarkers	Description, comments
CRD biomarkers used to guide AIT prescription	
Serum specific IgE antibodies to rPhl p 1, rPhl p 2, rPhl p 5, rPhl p 6	Molecular specific biomarkers of genuine sensitization to *Poaceae* grass pollen
Serum specific IgE antibodies to nCyn d 1	Molecular specific biomarkers of genuine sensitization to *Chloridoideae* grass pollen
Serum specific IgE antibodies to CCDs	Molecular biomarkers of sensitization to CCDs involved in specific IgE assays cross-reactivity
Serum specific IgE antibodies to rPhl p 7	Molecular biomarkers of sensitization to pollen polcalcin panallergens cross-reactive with pollen from most plants
Serum specific IgE antibodies to rPhl p 12	Molecular biomarkers of sensitization to pollen profilin panallergens cross-reactive with pollen, some plant-derived foods and latex
Predictive candidate biomarkers of AIT clinical efficacy	
Stabilin-1 (intracellular scavenger receptor), C1Q complement component expression	Intracellular biomarkers of tolerogenic dendritic cells
Coregulatory PD-L1 (B7-H1, CD274) expression	Surface cell biomarker of tolerogenic antigen presenting cells
Peripheral IL-10$^+$Foxp3$^+$ cells proportion among CD25$^+$ CD4$^+$ leukocytes	Regulatory T cell biomarker
Serum allergen-specific IgE to total IgE ratio	Allergen-specific antibodies biomarkers
Serum allergen-specific IgG_4, IgG_1 and IgA_2	
Inhibition of CD23-dependent IgE-FAB to B cells, serum specific IgE-BF competing with IgE for allergen binding	Functional biomarkers of serum IgG-associated inhibitory activity
Serum neopterin and kynurenine-tryptophan ratio	Molecular biomarkers of T cell mediated immune activation
Serum sHLA-G	Non-classical MHC class I immune tolerance molecular biomarker
Serum sTRAIL	TRAIL biomarker

CRD: Component-resolved diagnostic; CCDs: Carbohydrate cross-reactive determinants; IgE-FAB: IgE-facilitated allergen binding; IgE-BF: IgE-blocking factor; sHLA-G: Soluble HLA-G; sTRAIL: Soluble tumor necrosis factor-related apoptosis-inducing ligand; AIT: Allergy immunotherapy; PD-L1: Programmed death ligand-1; MHC: Major histocompatibility complex.

Table 3 Grasses (*Poaceae* family) which are sources of the most allergenic pollen grains[31,32,47]

Subfamily	Tribe	Species (common names)
Pooideae	*Poeae*	*Phleum pratense* (Timothy grass, Herd's grass, meadow cat's-tail)
		Lolium perenne (perennial ryegrass)
		Dactylis glomerata (orchard grass or cock's foot grass)
		Poa pratensis (smooth meadow grass or bluegrass)
		Anthoxanthum odoratum (sweet vernal grass)
		Festuca pratensis syn. *F. elatior* (meadow fescue)
		Holcus lanatus (velvet grass or Yorkshire fog)
		Agrostis capillaris syn. *A. tenuis or A. vulgaris* (bent grass)
		Arrhenatherum elatius syn. *Helictotrichon elatius* (tall oat grass)
		Avena fatua (wild oat)
		Avena sativa (cultivated oat)
	Triticeae	*Hordeum vulgare* (barley)
		Secale cereale (rye)
		Triticum aestivum ssp. *vulgare* (cultivated bread wheat)
	Bromeae	*Bromus inermis* (smooth brome)
Chloridoideae	*Cynodonteae*	*Cynodon dactylon* (Bermuda grass, Bahama grass, Devil grass)
Panicoideae	*Andropogoneae*	*Sorghum halepense* (Johnson grass)
	Paniceae	*Paspalum notatum* (Bahia grass)

subtropical grasses, such as *Cynodon dactylon* and *Paspalum notatum*[32,47,48].

Monitoring pollen in the air, carried out by various gravimetric, impaction and suction sampling devices, may be used for the management of pollen allergy, and for biomedical and biological research. The Hirst trap and later modified Burkard or Lanzoni traps are widely used samplers. Counting and identifying pollen grains is performed by optical microscopy. Pollen calendars are created based on differences in airborne pollen recorded in time[49]. Although pollen is routinely monitored, it is unknown whether pollen counts represent allergen exposure because pollen grains can vary substantially in allergen release, even although they are morphologically identical. There is a switch of importance from pollen count to pollen potency in the modern molecular era of aerobiology[50,51]. Phenological studies reveal that airborne grass pollen results from both local and distant sources, although the pollen airborne concentration peaks usually appear when such local herbaceous plants are shedding the greatest amounts of pollen. Although there is an association between flowering phenology and airborne pollen records for some of the tree and weed pollen types, for *Poaceae* the flowering and airborne pollen peaks usually do not coincide, with up to one week difference in phase[52]. Moreover, diurnal variations, climate and weather changes impact pollen exposure. Meteorological factors (temperature, wind speed, humidity, rain, thunderstorms) along with climatological regimes (warm or cold anomalies, dryer and wetter periods) influence pollen distribution. Human activities increase atmospheric greenhouse gases, such as carbon dioxide, and induce changes in global climate. Over the last decades, high

Table 4 Grass pollen seasons timing and temporal overlap in Europe[32,55-59]

Regions	Grass pollen seasons timing and temporal overlap with other types of pollen
Northern, Western, Central and Eastern Europe	Grass flowering period starts at the beginning of May, finishes at the end of July Some grass plants are in flower even in August, even September (*e.g.*, Poland) Air concentration of grass pollen usually peaks in June; pollination occurs about two-three weeks earlier at sea level and thereafter in the mountain regions Birch (*Betula* spp): Western Europe flowering period starts at the end of March, Central and eastern Europe from early April until May (2-8 wk), Northern Europe from late April to late May Ash tree (*Fraxinus* spp): In Central and South-Eastern Europe flowering period may partially overlap (April to May) with grass pollen season *Asteraceae* weeds, such as ragweed *Ambrosia artemisiifolia* var. *elatior* and mugwort *Artemisia vulgaris*, pollen season in Central and Eastern Europe may last from July to August-September Plantain *Plantago* spp: Pollen season from May to September in Eastern Europe
Mediterranean regions of Europe	Different grasses are flowering between April and August Olive (*Olea europaea*): Pollen season lasts from April to June, in regions of Greece, Spain, and southern Italy, overlapping grass pollen season Plane (*Platanus* spp): Pollen season partially overlapping with grass season, from April to May, in Southern France or Spain Pellitory *Parietaria* spp: Pollen has a long persistence in the atmosphere in the Mediterranean region, from April to October, even longer (perennial) *Asteraceae* weeds: Pollinate from August to October, similar to *Chenopodiaceae*/*Amaranthaceae* pollen from salt-tolerant weeds significant also for semi-arid areas Plantain *Plantago lanceolata*: Pollen season from April to July in Northern Spain
European islands with special climate characteristics	Iceland (cold-temperate oceanic country): Some grass species and sorrel (*Rumex* spp) flower in June, both with peaks in July; a second peak of grass-pollen is possible in some years in August; pollen season tails off in September; birch pollen season is short, starting in the second part of May until the beginning of June Canary Islands (Spanish archipelago with subtropical climate): Long-range transport of *Poaceae* and *Amarantaceae*/*Chenopodiaceae* pollen from southern Iberian Peninsula and Morocco (mixed with *Oleaceae* tree pollen) and from the African Saharan sector and Sahel

Table 5 Grass pollen seasons timing and temporal overlap in Africa[60-65]

Regions	Grass pollen seasons timing and temporal overlap with other types of pollen
Extremely variable pollen seasons exist due to great differences in plant distribution	
Morocco (North Africa)	Mediterranean region: grass pollen season starts in April and has highest air pollen concentration in May and June, overlapping olive pollen season Date palm (*Phoenix dactylifera*) from *Arecaceae* family is distributed not only in the Mediterranean areas of Morroco, but also Middle East and central Africa
Nigeria (West Africa)	Southwest region: at the end of the dry season, in March, airborne pollen grains of *Chenopodiaceae*/*Amaranthaceae* and *Poaceae* coexist in significant concentrations
South Africa	Grass pollinating season peaks from October to February (longer grass flowering); in subtropical regions, grasses of the subfamily *Panicoideae* are predominant: *Cenchrus clandestinum* (kikuyu) and *Stenotaphrum secundatum* (buffalo grass) South African *Cupressaceae* (cypress) trees start flowering in June, followed by *Quercus robur* (oak) in late July, *Platanus* (plane) in September and *Olea europaea* subsp. *africana* (olive) in January, and because their pollen season duration lasts three to four months it overlaps the grass pollen season *Plantago lanceolata* (English plantain) and *Chenopodiaceae* (goosefoot) are most important weeds with pollen seasons in late summer and autumn, overlapping with the grass pollen season

temperatures and atmospheric carbon dioxide concentration have impacted plant and pollen distribution and induced changes in quantitative production and dispersion of pollen, pollen seasons and allergen content of pollen grains, which are region and species-specific[32,53,54].

Grass pollen seasons timing and temporal overlap with other types of pollen must be discussed for different regions in the world (Tables 4-8). Diagnostic molecular biomarkers represented by specific IgE against recombinant allergen components, are especially important in patients with multiple sensitizations to different pollen types within this context.

As is inferred from the presented data, grass species produce the only allergenic pollen with ubiquitous representation and clinical significance across the globe. In many regions, grass pollen seasons overlap other pollination periods of other anemophilous plants (trees and weeds); therefore, commercially marketed CRD assays for inhalant sources include grass pollen allergens[26].

Serum levels of specific IgE to recombinant and native allergen components (specific and cross-reactive pollen allergen components) can be measured *in vitro* using two types of tests. Singleplex diagnostic tests (one result for a single serum specimen) are the same immunoassays as those used for the IgE determinations for allergenic extracts, the difference being that the antigen is a highly purified molecule, either natural or recombinant. Multiplex diagnostic tests (several results for a single speci-

Table 6 Grass pollen seasons timing and temporal overlap in Asia[32,66-84]

Regions	Grass pollen seasons timing and temporal overlap with other types of pollen
Western Asia, Middle East	Most Middle East countries: significant grass and weed pollen seasons during April to May and September to October Desert and semi-desert countries: *Chenopodiaceae* pollen season overlapping with grass pollen season, such as for *Cynodon dactylon* Indigenous trees/shrubs, such as mesquite (*Prosopis juliflora*) and date palm (*Phoenix dactylifera*) pollen seasons from March to May, also in Egypt Israel: *Cupressaceae* (cypress family) and *Poaceae* pollen seasons throughout the year, grasses especially in spring (March-May), cypresses February and April; *Olea europaea* flowering begins in late March till July-August; *Parietaria judaica* pollen highly allergenic in northern Israel Turkey Mediterranean coastal area (Antalya): grass pollen frequently detected between April-May and October-November and found in the atmosphere in high concentrations during May to July; *Pinaceae* pollen airborne between March and June; *Cupressaceae* pollen, in high levels in February, usually present until May; *Chenopodiaceae*/*Amaranthaceae* pollen grains found in air during June to October, all overlapping with the grass pollen season
South Asia	Grass pollen season overlaps with seasons of many other anemophilous plants Pakistan: paper mulberry (*Broussonetia papyrifera*, family *Moraceae*) pollen season from March to April overlaps with the grass pollen season India: spring (February-April), autumn (September-October), winter (November-January) pollen seasons include periods of flowering of grasses (*Cynodon dactylon, Paspalum distichum, Sorghum vulgare, Poa annua*), of weeds *Cannabaceae* (*Cannabis sativa*), *Asteraceae* (*Parthenium, Artemisia* spp), and *Chenopodiaceae* (*Amaranthus*, *Chenopodium* spp), and of trees (*Prosopis juliflora*, *Cocos* and *Eucalyptus* spp)
East Asia	Beijing and different provinces of the People's Republic of China, such as Guangdong, Yunnan and Hebei: pollen season lasting from August to October is due to weed pollen from different plant families, *Chenopodiaceae*, *Asteraceae* (mugwort *Artemisia* spp), *Cannabaceae* (hop *Humulus* spp), but also to grass pollen South Korea: grass pollen airborne between end of April and November, especially Korean lawn grass, Timothy grass, Bermuda grass, and orchard grass; Pollen seasons of trees (pine, birch, oak) and weeds (mugwort, ragweed, Japanese hop) overlap with grass pollination period in the first, respectively last part of it Japan: pollen season for Japanese cypress/hinoki (*Chamaecyparis obtusa*) lasts from March to May, while for orchard grass (*Dactylis glomerata*), in May-June to August, and for weed yomogi *Artemisia*, from August to October Olive pollen from May to June overlaps with orchard grass season in Shodoshima *Plantago lanceolata* pollen dispersed from mid-May to early September, in Sapporo

Table 7 Grass pollen seasons timing and temporal overlap in America[28,85-93]

Regions	Grass pollen seasons timing and temporal overlap with other types of pollen
United States of America and Canada (North America)	Temperate regions: tree pollen predominates in spring, grasses in late spring and early summer, and weeds from summer until fall, with variable overlap periods Ragweed (*Ambrosia* spp) pollen season starts in July and peaks between August and October, warming by latitude being associated with increased length of pollen season in central North America Mountain cedar (*Juniperus ashei*) pollen season, in Oklahoma, Arkansas, central Texas, lasts from December to February, not overlapping with grass pollen period Southern Texas, on the western Gulf Coast: airborne grass pollen concentrations have two peaks, one in May (due to cool temperate grass species) and one in September and October (due to temperate and subtropical species), long distance dispersal of grass pollen is possible also out of season Subtropical regions, such as Southern California, Florida: grass pollen season dispersed all year overlaps with *Cupressaceae* trees (December through May), *Asteraceae*, *Chenopodiaceae* weeds pollen seasons (usually May through December)
Mexico, Central and South America (Latin America)	Subtropical regions in Mexico: similar to Southern California and Florida Mesquite (*Prosopis* sp) pollen in Northern Mexico and Southwestern United States Tropical regions: grass pollen grains airborne throughout the year, overlapping with the pollination periods of trees, such as *Anacardiaceae*, *Cupressaceae*, and weeds, such as *Amaranthaceae*, *Asteraceae* and *Euphorbiaceae* spp Maule region of Chile: *Platanus acerifolia*, *Olea europaea*, *Cupressus* spp pollen and grass pollen detected in August through November until end of January; *Plantago* spp, *Rumex* and *Chenopodium* spp pollen present from October to April

Table 8 Grass pollen seasons timing and temporal overlap in Australasia[48,94-98]

Regions	Grass pollen seasons timing and temporal overlap with other types of pollen
Australia	Subtropical northern regions (Brisbane, Queensland): grass pollen season, such as for *Paspalum notatum*, *Sorghum halepense* and *Cynodon dactylon*, from summer to autumn months, December to April, overlaps with the pollen season of groundsel bush (*Baccharis halimifolia*, *Asteraceae* family) Oceanic southern regions (Melbourne, Victoria): temperate grasses pollinate especially in spring, from September to November, overlapping with trees *Cupressus* and *Betula* spp pollen season
New Zealand	Temperate grasses form the major component of atmospheric pollen levels during spring and summer (October to February) and *Plantago* spp pollen season overlap

men) are immuno solid-phase allergen chip based on multiplex microarray-based technology, multiparameter immunoblot test system based on single purified allergen components, and a multiplex flow cytometry allergenic molecule-based micro-bead array system[45,99-103].

In contrast to traditional specific IgE biomarkers, CRD in allergy does not rely upon whole extract preparations from native allergen sources, but on quantification of specific IgE antibodies to single protein components, purified from natural sources (native allergen components) or obtained using recombinant techniques (recombinant allergen components). These modern diagnostic biomarkers are useful for a detailed CRD of the sensitization and cross-reactivity profiles, discriminating between clinically significant and irrelevant specific IgE, reduce the need for provocation testing and improve the prescription and specificity of AIT[26,45,104,105].

Molecular specific biomarkers of genuine sensitization to grass pollen

Molecular and biochemical characterization of grass pollen reveals several important specific allergen components. Timothy grass (*Phleum pratense*), also known as Herd's grass, meadow cat's-tail or common cat's tail, belongs to the *Pooideae* subfamily and it is one of the most significant source of grass pollen allergens in temperate regions. Bermuda grass (*Cynodon dactylon*), also known as Scutch grass, Bahama grass, Devil grass, belongs to the *Chloridoideae* subfamily, and it is an important grass which typically grows in warm temperate, subtropical and tropical climates areas of the world.

Specific IgE antibodies to recombinant temperate grass-specific pollen allergen components, rPhl p 1, rPhl p 2, rPhl p 5 and rPhl p 6, are biomarkers of genuine sensitization to *Poaceae* pollen. From references[26,33,106-124], these specific components and correspondent antibody biomarkers are discussed below.

Phl p 1 belongs to the group 1 grass pollen allergens, acidic glycoproteins with molecular mass of 31-35 kDa, a family of major allergens present in all grass species (*Poaceae* family-specific marker). More than 90%-95% of grass pollen allergic patients, adults or children, have specific IgE to group 1 grass pollen allergens. Group 1 grass pollen allergens are glycosylated proteins that show 60%-70% sequence identity to beta-expansin family of cell wall-loosening proteins with a role in pollen tube penetration into the style and pollen tube growth. A major IgE-reactive domain of Phl p 1 exhibits significant sequence identity of 43% with the family of immunoglobulin domain-like group 2/3 grass pollen allergens. Recombinant Phl p 1, rPhl p 1 (27 kDa) is not glycosylated and resembles native Phl p 1 (nPhl p 1) closely binding to IgE in about 90% of patients with grass pollen allergy, revealing that rPhl p 1 shares many of the IgE epitopes with natural grass allergens of the group 1. Sensitization to rPhl 1 seems to appear earlier in life in comparison with other allergen components. Group 1 grass pollen allergens with great sequence identities and homologies include, besides Phl p 1, other important allergen components from important grass pollen grains: *Anthoxanthum odoratum* (Ant o 1), *Dactylis glomerata* (Dac g 1), *Holcus lanatus* (Hol l 1), *Lolium perenne* (Lol p 1), *Poa pratensis* (Poa p 1). There is a partial cross-reactivity between Phl p 1 and Cyn d 1, the group 1 major allergen in Bermuda grass *(Cynodon dactylon)*, thus Phl p 1 is only partially specific for the *Pooideae* grass subfamily.

Phl p 5 is another major allergen from Timothy grass pollen and is one of the most reactive of the group 5 allergens, ribonucleases generally restricted to the *Pooideae* subfamily of grass pollen. Between 65%-90% of grass pollen allergic patients in temperate climate areas are sensitized against group 5 grass pollen allergens components. Grass pollen grains in ambient air is not quantitatively correlated with the airborne Phl p 5 concentration. Rainfall contributes to an increase in respirable particles containing group 5 allergens, which bursts the pollen grains. Moreover, exposure of pollen to gaseous pollutants induces a decrease in Phl p 5 detection in pollen extracts due to a mechanical loss of allergens from the altered pollen grains and/or post-translational modifications, such as ozone acidification. Phl p 5b, a smaller isoform (32 kDa), contains at least one more IgE antibody binding epitope than Phl p 5a isoform. rPhl p 5 is very similar to nPhl p 5 and reacts with serum IgE antibodies in a great part of grass pollen-allergic patients. rPhl p 5 is cross-reactive with similar group 5 allergen components: Dac g 5, Lol p 5, Poa p 5, Ant o 5. Because group 5 allergens are restricted to the *Pooideae* subfamily, there is a limited cross-reactivity between the pollen of temperate-type *Pooideae* subfamily grasses and pollen from warm temperate/subtropical-type grasses belonging to *Chloridoideae* (*Cynodon dactylon*) and *Panicoideae* (*Paspalum notatum*) subfamilies. Common reed (*Phragmites communis*), a grass from the *Arundinoideae* subfamily with a low phylogenetic affinity to *Pooideae* plants, produces pollen in late summer to autumn with a very low degree of cross-reactivity to group 5 allergens. There is a dissociation of the major IgE and T-cell-reactive peptide domains in Phl p 5. Specific IgE antibodies against Phl p 1 and Phl p 5 might be used as a reliable biomarker of allergy to *Poaceae* pollen. These major allergen components are defined on the basis of both frequency (prevalence of specific IgE antibodies) and potency (average level of specific IgE antibodies). Mono-sensitization to rPhl p 1 seems important in patients with lower IgE against Timothy grass pollen extract levels, while sensitization to rPhl p 5 is rarely found as the only sensitizing allergen.

Other grass-specific pollen allergen components must be discussed. IgE to rPhl p 2 (13 kDa) may also be regarded as a fairly specific biomarker for patients sensitized to grass species of the *Pooideae* subfamily. Immunologically significant group 5 and group 2 allergens seem to be absent in non-*Pooideae* grass pollen grains. Phl p 6 (a group 6 acidic, nonglycosylated protein of 15 kDa, for which N-terminal sequencing reveals homology to an internal region of group 5 allergens), along with Phl p 5,

do not exhibit significant serological cross-reactivity to pollen allergens outside the *Pooideae* subfamily. rPhl p 6, with the same reactivity with serum IgE antibodies as the native molecule, can be used for *in vitro* diagnosis of grass pollen allergy.

In conclusion, specific IgE against rPhl p 1 is a *Poaceae* family-specific biomarker for genuine sensitization to grass pollen and specific IgE antibodies against rPhl p 2, rPhl p5 and rPhl p 6 are *Pooideae* subfamily-specific biomarkers for true sensitization to temperate grass pollen. rPhl p 1, rPhl p 5 and natural Timothy extract are used to identify grass pollen allergy. Mono/oligo-sensitized patients with specific IgE to non-glycosylated major species-specific allergen markers (Phl p 1, Phl p 5) are suitable for *Pooideae* grass-specific AIT[26,33,117,123].

Specific IgE antibodies to nCyn d 1, a warm climate grass-specific native pollen allergen component, represent biomarkers of genuine sensitization to *Chloridoideae* subfamily grass pollen, as discussed below[26,125-129]. Cyn d 1 is a major allergen most abundant in Bermuda grass pollen, representing 15% of the whole-pollen extract. The frequency of sensitization to Cyn d 1 in Bermuda grass-allergic individuals is between 76% and 100%. Cyn d 1 belongs to Group 1 grass pollen allergens, including highly cross-reactive pollen allergens from other *Chloridoideae* subfamily grasses, such as Bou g 1 from the pollen of the North American Grama grass (*Bouteloua gracilis*). Cyn d 1 is to some extent immunologically distinct from Phl p 1 from Timothy grass and therefore a suitable marker for sensitization to *Cynodon dactylon*. Partial cross-reactivity between Phl p 1 and Cyn d 1 may impede the identification of the sensitizing allergenic source. When testing for rPhl p 5 as a *Pooideae*-specific molecular biomarker is negative, relatively higher levels of IgE specific to nCyn d 1 than to rPhl p 1 have been suggested to be indicative of primary sensitization to Bermuda grass pollen, an AIT extract containing *Cynodon dactylon* pollen might be suitable. If testing for IgE, anti-rPhl p 5 is positive and specific IgE against nCyn d 1 higher than to rPhl p 1, there is a true double sensitization. Finally, if antibodies against *Pooideae*-specific molecules, such as rPhl p 5, are positive and specific IgE levels against rPhl p 1 have higher levels than those to nCyn d 1, the case is most probably primary sensitization to *Pooideae* grasses and *Cynodon dactylon* pollen representation can be omitted from the AIT regimen.

Specific IgE antibodies to recombinant and native specific allergen components from tree and weed pollen are important to differentiate the true sensitization profile in patients with multiple sensitizations, including grasses, as described below[26,47,129-139]. When testing for these specific pollen components is negative and testing for IgE against specific and cross-reactive grass allergen components, then IgE sensitization is to grass pollen. If testing for IgE against recombinant specific grass pollen components is positive and specific IgE against specific tree or weed components are also significant, the condition is a true double or multiple sensitization.

Tree pollen-specific allergen components are described for the anemophilous plants belonging to the *Betulaceae* family: rBet v 1, a 17 kDa pathogenesis-related protein PR-10 with ribonuclease activity from the pollen of silver birch *Betula pendula* or *Betula verrucosa*, cross-reactive with other *Betulaceae* pollen PR-10 components with about 70% identity to Bet v 1 (black alder *Alnus glutinosa* rAln g 1, hazel *Corylus avellana* rCor a 1.0101); *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, 43 kDa pectate lyase from the pollen of Arizona cypress *Cupressus arizonica*, cross-reactive with other *Cupressaceae* pollen pectate lyase components (Japanese cedar *Cryptomeria japonica* nCry j).

Major native or recombinant weed pollen-specific allergen components are described for herbaceous weeds 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*; family *Plantaginaceae*: rPla l 1, a 17 kDa Ole e 1-like trypsin inhibitor from the pollen of plantain *Plantago lanceolata*; family *Urticaceae*: rPar j 2, a 14 kDa lipid transfer protein, member of the PR-14 protein family, from the pollen of wall pellitory *Parietaria judaica*; family *Amaranthaceae/Chenopodiaceae*: 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*.

Molecular biomarkers of sensitization to carbohydrate cross-reactive determinants

Carbohydrate cross-reactive determinants (CCDs) are carbohydrate moieties of glycoproteins that induce the production of highly cross-reactive IgE, as discussed below[26,140-143]. Many allergens are glycoproteins containing carbohydrate moieties called N-glycans or O-glycans, according to their site of attachment to the protein. N-glycans containing beta1,2-xylose and alpha1,3-fucose in many glycoproteins are more extensively studied. Markers of sensitization to CCDs are bromelain (nAna c 2) and MUXF3 (Ana c 2.0101) carbohydrate epitope, the purified N-glycan from *Ananas comosus* bromelain, able to detect IgE to N-glycans in most pollen sources. Anti-CCD IgE biomarkers indicate the presence in serum of IgE directed against carbohydrate epitopes. CCDs rarely cause allergic reactions, but may produce positive *in vitro* test results to CCD-containing allergens from pollen, plant foods, insects and venoms. Patients sensitized to grass pollen develop anti-CCD IgE that also binds to CCD monovalent peanut allergens, but does not induce any clinical symptoms. Approximately 20% of patients with multiple pollen allergies have IgE antibodies to pollen allergens with molecular masses higher than 30 kDa and a great part of their IgE-binding is dependent on CCDs,

a major cause of cross-reactivity for *in vitro* specific IgE assays. If testing for IgE against a specific native allergen component, such as nCyn d 1, is positive, because native components are CCD-containing natural purified glycoproteins, it is necessary to assess the epitope protein nature in multi-sensitized patients. In cases of positive *in vitro* results to a natural allergen component, negative IgE to CCD markers reveal the protein nature of IgE epitopes. Positive IgE to CCD markers should optimally be accompanied by assessment of biological activity, such as positive skin prick testing or nasal/conjunctival challenge with the allergen, important aspects in the AIT decision process.

Molecular biomarkers of sensitization to cross-reactive pollen panallergens

Panallergens, usually classified as minor allergens, are defined as homologous and structurally related proteins belonging to different biological sources and causing IgE cross-reactivity between evolutionary unrelated species. Among panallergen families, only profilins are distributed ubiquitously throughout the plant kingdom and are responsible for allergic reactions to a multitude of evolutionary unrelated pollen and food allergen sources. Occurring exclusively in pollen grains of plants, polcalcins are not involved in pollinosis-associated plant food allergies. Bet v 1 homologues represent major allergens in pollen of trees *Fagales* (including the *Betulaceae* and *Fagaceae* families) but can also be found in many allergenic foods belonging to the botanical families of *Rosaceae* (PR-10 proteins with 50%-60% identity to Bet v 1: apricot Pru ar 1, plum Pru c 1, peach Pru p 1, cherry Pru av 1, apple Mal d 1, pear Pyr c 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), giving rise to many birch pollinosis-associated food allergies. Bet v 1-like allergens are not normally present in the pollen of grasses or weeds[132,144,145]. Although AIT with the recombinant major birch pollen allergen Bet v 1 proved as efficient as purified native Bet v 1 or birch pollen extract[22,146], the presence of IgE-sensitization to minor allergen components acting as panallergens, profilins and/or polcalcins, would be expected to decrease the efficacy of pollen AIT, at least to some extent, especially in the absence of IgE to species-specific allergen components. Sensitization to both profilin and/or polcalcin typically follows previous cosensitization to other molecular allergens from the same pollen source, being recognized at a later stage, and it is associated with a longer duration of allergic disease and with resulting cosensitization to a larger number of species-specific allergen molecules. When molecular multi-sensitization is present, sometimes it is associated with the practical inability to administer a more appropriate, allergen-matching AIT extract. Even if the content in various pollen AIT extracts, at least for profilin, is remarkably low, if specific IgE antibodies against major allergens are present, AIT with extracts containing these allergens can be administered, especially as the clinical relevance of profilins and polcalcins is still arguable[26].

Only a limited number of pollen panallergens are available for routine use (grass profilin, rPhl p 12, and birch profilin, rBet v 2; grass polcalcin, rPhl p 7 and birch polcalcin, rBet v 4), but due to marked structural homology among allergenic species, these serve as efficient markers of IgE-mediated hypersensitivity to the entire group of homologous proteins, with the possible exception of profilins from pollen of wall pellitory *Parietaria judaica* (Par j 3) and cypress *Cupressus sempervirens* (Cup s 8), the latter being cross-reactive with the goosefoot *Chenopodium album* profilin, Che a 2. The molecular biomarkers of sensitization to cross-reactive grass pollen panallergens are discussed below[26,117,140,145,147,148].

rPhl p 7, a 9 kDa calcium-binding protein, is used as a polcalcin marker. Phl p 7 is a minor allergen of Timothy grass pollen, recognizing serum IgE antibodies in 10%-15% of grass pollen-sensitized subjects. Phl p 7 is a polcalcin cross-reactive with other polcalcins contained in pollen grains of non-*Pooideae* Bermuda grass (Cyn d 7), trees, such as birch (Bet v 3), alder (Aln g 4), olive (Ole e 3), juniper (Jun o 4), and weeds, such as goosefoot (Che a 3). Unlike Bet v 3 which contains three typical calcium-binding motifs, Bet v 4 is a polcalcin which contains only two calcium-binding domains. rBet v 4, a 8 kDa calcium-binding protein, is also used as a polcalcin marker. Other weed pollen polcalcins are from *Asteraceae* family (Art v 5, Amb a 10). Polcalcin rPhl p 7 is therefore likely to cross-react with pollen proteins from most plants, in particular with other grass species, several weeds and trees.

rPhl p 12, a 14 kDa actin-binding protein, is used as a profilin marker. This acidic protein is involved in cytoskeleton dynamics by binding to actin. Phl p 12 is a minor allergen of Timothy grass pollen, binding IgE antibodies from approximately 15%-30% of grass pollen-allergic subjects with varying degrees in different geographical regions. Phl p 12 has more than 75% sequence identity with profilins from pollen, various plant-derived foods and latex. It is cross-reactive with pollen profilins from many plants, such as birch (Bet v 2), olive tree (Ole e 2), date palm (Pho d 2), Bermuda grass (Cyn d 12) and sunflower (Hel a 2). rBet v 2, a 15 kDa profilin, is also used as a cross-reactive marker. Other pollen profilins are those from ragweed (Amb a 8) and mugwort (Art v 4). 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 the celery-mugwort-spice syndrome. Cross-reactivity between profilins of ragweed pollen (Amb a 8) and fruits, such as melon (Cuc m 2) and banana (Mus xp 1), are involved in the pathogenesis of the ragweed-melon-banana association.

Molecular diagnosis biomarkers, together with clinical history data, can help clinicians make a better selection of the most appropriate patients and allergens for AIT[140]. Moreover, application of the component-resolved diagnosis biomarkers may change the diagnosis and the

choice of AIT in some patients[149].

Taken together, the CRD biomarkers are used to guide prescription of grass pollen AIT after an initial basic diagnostic discrimination between mono/oligo- and multi-sensitization, based on skin prick testing results and/or values of *in vitro* evaluation of specific IgE using common pollen extracts. The use of a panel of species-specific allergen molecular markers, representing the most common allergenic species in the region, along with the panallergen screening molecules from grass pollen (polcalcin rPhl p 7 and profilin rPhl p 12), may facilitate the selection of those AIT candidates with an increased probability of benefiting from this type of treatment.

PREDICTIVE BIOMARKERS OF CLINICAL EFFICACY

In grass pollen immunotherapy

Because very complex immunological mechanisms of action, both cellular and humoral, are involved in the AIT efficacy, its long-lasting effect and the way it changes the course of IgE-mediated allergic disease, candidate biomarkers of clinical efficacy or biomarker combinations remain to be validated in order to clearly distinguish between strong and weak or early and late AIT responders[42].

The AIT mechanisms of action to induce peripheral tolerance to grass allergens may be useful to classify some candidate *predictive biomarkers* for AIT efficacy, especially those derived from the antigen presenting cell (APC)-regulatory T cell (T_{reg})-IgG_4 antibody immunoregulatory loop[150]. These candidate biomarkers can be classified as tolerogenic DCs biomarkers, regulatory T cell biomarkers, serum blocking antibodies biomarkers, especially functional ones, immune activation and immune tolerance soluble biomarkers and apoptosis biomarkers[39-42,44].

Biomarkers of tolerogenic DCs

Oral APCs are key players in SLIT. Langerhans cells, $CD207^+$ cells (Langerin or CD207 being a C-type lectin receptor localized in Birbek granules) located in the mucosa itself, with a $Fc_{epsilon}RI$ expression greater compared with similar cells in the skin[151], and a predominant subpopulation of myeloid DCs located along the *lamina propia*, $CD11b^+CD11c^-$ monocyte-derived DCs (moDC), are critical in capturing allergen and processing it as small peptides presented in association with major histocompatibility complex (MHC) class I and class Ⅱ molecules at the cell surface. DCs loaded with allergen-derived peptides migrate to the cervical lymph nodes within 12-24 h, where they interact with naive $CD4^+$ T cells to support the differentiation of T_{reg} cells within 2-5 d. These $CD4^+$ T cells subsequently migrate through blood back to mucosal tissues, resulting in allergen tolerance associated with downregulation of Th_2 responses[152,153].

Intracellular and surface biomarkers of tolerogenic DCs are important to be presented.

Biomarkers of tolerogenic DCs (DC_{reg}) are biomarkers of DCs driving differentiation of T_{reg} cells, evidenced by differential gel electrophoresis and mass spectrometry[154]. Two such biomarkers must be discussed. Stabilin-1 (STAB1) is an intracellular scavenger receptor expressed by DCs and macrophages. Complement component 1 (C1Q) is the first component of complement which may be associated with arrest of moDC differentiation and may induce tolerogenic properties in developing DCs[154-156]. Tolerogenic moDCs are the most prominent source of *C1Q* and *STAB1* gene expression in the blood and are generated *in vitro* from peripheral blood mononuclear cells (PBMCs). Induction of DC_{reg} biomarkers (DCs *in vitro* treatment with dexamethasone) in PBMCs (containing < 0.5%-1% DCs) of patients with grass pollen allergy treated four months with SLIT is indicative of clinical tolerance induced by AIT (short-term efficacy)[154].

Regarding surface biomarkers of tolerogenic DCs, SLIT downregulates APC functions by modulating the expression of costimulatory molecules. There is a recent role revealed for the programmed death-1 receptor (PD-1) and PD-1 ligand (PD-L1) pathway in regulating lymphocyte activation and promotion of T_{reg} cell development and function[157]. PD-L1 (B7-H1, CD274), the programmed death ligand-1, is a coregulatory molecule critical for T_{reg} generation with important expression on tolerogenic APCs (upregulated by TLR4 ligand monophosphoryl lipid A). PD-L1 may play an important role in induction of T regulatory cells by SLIT[158]. Pollen SLIT reduces the expression of CD86 on B cells ($CD19^+$) and the expression of CD80 on monocytes ($CD14^+$), and increases the expression of PD-L1 on APCs ($CD14^+$, $CD19^+$) evaluated by flow cytometry analysis. PD-L1 may be a major target of pre-seasonal pollen SLIT and that modulation of its expression could be used as a clinical efficacy marker[150].

Regulatory T cell biomarkers

These biomarkers may also be important because multiple mechanisms are related to T_{reg} cells in AIT. T_{reg} cells directly and indirectly control the activity of effector cells of allergic inflammation, such as eosinophils, basophils and mast cells. AIT-induced T_{reg} cells inhibit the $Fc_{epsilon}RI$-dependent mast cell degranulation, OX40-OX40 ligand interaction playing an important role, decrease the thresholds for mast cell and basophil activation and reduce IgE-mediated histamine release[159-163]. Both main subsets, naturally occurring forkhead box P3 (FoxP3) expressing $CD4^+CD25^+$ regulatory T cells and inducible IL-10-producing T regulatory type 1 (Tr_1) cells, are decisive for the development of immune tolerance to allergens under AIT[163]. Mucosal T_{reg} cell induction in SLIT was revealed by immunofluorescence microscopy, $FoxP3^+$ cells being increased in the oral epithelium of grass pollen SLIT[164]. The induced T_{reg} cell level defined as the proportion of $IL\text{-}10^+FoxP3^+$ cells among $CD25^+CD4^+$ leukocytes, analyzed in the peripheral blood by flow cytometry, may be a potential therapeutic biomarker for SLIT, as revealed in a preliminary report in Japanese cedar (*Cryptomeria japonica*) pollinosis[165]. Allergen-specific $CD4^+$ T cell responses in

peripheral blood do not predict the early onset of clinical efficacy during grass pollen SLIT, as revealed in a more recent study in which these peripheral allergen-specific $CD4^+$ T cells were assessed using pMHCII-tetramers or flow cytometry surface phenotyping, as $CTLA\text{-}4^+IL\text{-}10^+$ or $CD25^+CD127^-FoxP3^+$ T_{reg} cells. Moreover, transcription factors (GATA-3, FoxP3) and cytokines (TGF-beta) gene expression assessed by quantitative reverse transcriptase polymerase chain reaction in allergen-stimulated peripheral cells do not predict clinical efficacy in SLIT, and the downregulation of *IL-4* or *IL-10* gene expression, as well as IL-10 secretion, by allergen-stimulated T cells seems to be unrelated to clinical benefit[166].

Antibodies biomarkers

The candidate antibodies biomarkers for the prediction of efficacy and monitoring of grass AIT must be discussed correlated with the allergen-specific IgE and IgG_4 responses during AIT.

Serum allergen-specific IgE antibodies

Although AIT rapidly induces peripheral T-cell tolerance, B-cell changes seem to appear at a relatively later phase. Serum allergen-specific IgE values are not generally considered appropriate biomarkers to assess SIT efficacy. Sometimes they transiently increase early in SCIT, and then gradually decrease over months or years of continued treatment. In pollen-sensitive patients who have undergone AIT and become desensitized, these values do not increase during the pollen season. There is a blunting of seasonal increases in specific IgE antibodies by AIT. Very late in the course and after termination of AIT, a decrease of allergen-specific IgE values is possible, occurring one to three years after starting therapy. Changes in IgE levels cannot account for reduced responsiveness to specific allergens after AIT because the decrease in serum IgE levels is late, relatively small and poorly correlated with efficacy. The reason for the persistence of serum IgE despite clinical improvement may relate to long-lived bone-marrow-resident IgE producing plasma cells[16,40,163,167,168].

The ratio of allergen-specific IgE to total IgE (sIgE/tIgE) was proposed as a candidate prognostic biomarker for SLIT. Symptom-medication score in patients treated with pollen SLIT seems to be correlated with the sIgE/tIgE ratio before treatment, being significantly improved in patients with a low sIgE/tIgE ratio compared to that in patients with a high sIgE/tIgE ratio. The grass-specific IgE to total IgE ratio seems significantly higher in responders than in nonresponders following four years of pollen SLIT. Further validation studies are needed before this biomarker can be considered in the clinical management of SLIT[158,169].

Serum allergen-specific IgG_4 antibodies

IgG_4 blocking antibodies prevent allergen-induced IgE-mediated release of inflammatory mediators from basophils and mast cells, directly compete with IgE on mast cells and APCs, inhibit IgE-facilitated allergen presentation to T cells and allergen-induced IgE production during allergen exposure. There is also an IgG_4-dependent blocking of IgE binding to B cells. IgG_4 production is confined to human IL-10-producing regulatory B (BR1 cells or $CD73^-CD25^+CD71^+$ B cells)[40,163,170,171].

Regarding serum allergen-specific IgG_4 antibodies as biomarkers, only specific IgG_4 antibodies with high affinity and avidity are functionally relevant. Pollen specific IgG_4 may be evaluated by fluoro-enzyme immunoassay. Serum allergen-specific IgG_4 levels significant increase relatively early in SIT (weeks to months after AIT start), in an allergen-dose dependent manner (10-100-fold increase) and persist for up to two years after AIT discontinuation. Although this indicates a good immunological response to AIT, there are contradictory correlations with clinical improvement, there is no correlation with clinical outcomes (after up-dosing) and there is no common cut-off value for specific IgG_4 antibodies[163,170,172].

Basophil activation evaluation may be used to detect IgG blocking activity in AIT. Allergen-IgG_4 complexes bind to $Fc_{gamma}RIIb$ (low affinity IgG receptor) containing a cytoplasmic immunotyrosine inhibitory motif that counters immunoreceptor tyrosine-based activation motif signals from $Fc_{epsilon}RI$ (high-affinity IgE receptor). Phosphorylated $Fc_{gamma}RIIb$ mediates inhibition of $Fc_{epsilon}RI$ signaling, coaggregation of $Fc_{epsilon}RI$ with $Fc_{gamma}RIIB$ inhibits degranulation, although there is a controversial role of $Fc_{gamma}RIIb$ in mediated post-AIT serum inhibitory activity[173,174]. Basophil activation test by flow cytometry evaluating CD203c expression, an ecto-nucleotide enzyme associated with basophil activation and piecemeal degranulation, may be a candidate biomarker for AIT monitoring, as suggested by a Japanese cedar pollen allergy study revealing a reduction in CD203c expression post-AIT[175].

Functional biomarkers of serum IgG-associated inhibitory activity in AIT may be more useful surrogates of clinical response than serum IgG_4 levels.

The inhibition of CD23-dependent IgE-Facilitated Allergen Binding (IgE-FAB) to B cells assay evaluates the serum inhibitory activity for binding of allergen-IgE complexes on to B cells. It is performed incubating allergen-IgE complexes with an EBV-transformed B-cell line, complexes bound to CD23 on the surface of cells being detected by flow cytometry. Addition of serum from patients who have received AIT inhibits allergen-IgE complex binding to CD23 on B cells. The following formula may be used to calculate the percentage relative B cell binding: % relative allergen-IgE complex binding to B cells = (% IgE-FAB using indicator and immunotherapy serum/% IgE-FAB using indicator serum only) × 100. Pollen SCIT induces in grass allergic rhinitis patients time- and dose-dependent increases in antibody-associated serum inhibitory activity for IgE-FAB and increases in IgE-blocking factor (IgE-BF)[168].

Serum specific IgE-BF competing with IgE for allergen binding is determined using a wash assay, IgE measurement with a chemiluminescent immunoassay, and

no-wash assay, allowing non-IgE antibodies to interact with biotinylated allergens in competition with the solid-phase absorbed IgE antibodies. The (IgE binding in competition with non-IgE)/(IgE binding with remaining Igs washed away) ratio varies from 0 to 1 (no blocking antibodies induced). Successful grass pollen SCIT is associated with significant reduced allergen-IgE binding (IgE-FAB) and increased IgE-BF[168].

Whether such functional assays of inhibitory IgG_4 and IgE-BF will be validated as predictive biomarkers of clinical AIT efficacy in individual patients requires further detailed investigation.

Serum allergen-specific IgA_2 antibodies

Regarding IgA subclasses, IgA_1 is found in serum and produced by bone marrow B cells, while IgA_2 is made by B cells located in the mucosa. The development of mucosal immune tolerance is associated with the expression of immunoregulatory cytokines (IL-10, TGF-*beta*) and protective antibody subclasses (IgG_4 and IgA_2)[164]. Long-term grass pollen AIT seems to induce a selective IgA_2 subclass systemic response, which may reflect a local mucosal response. Serum Phl p 5-specific IgA_2 response to AIT is associated with nasal TGF-*beta* expression. Allergen-specific IgA_2 concentrations can be determined by sandwich enzyme-linked immunosorbent assay (ELISA) and the systemic specific IgA_2 response might also be surrogate biomarker of the clinical response to AIT[176].

All of these studies approaching various humoral immunological pathways involved in AIT efficacy may create a framework regarding the usefulness of *antibodies biomarkers*, but the mechanisms of grass pollen-specific IgG_4 and also IgG_1 antibody subclasses in AIT are not very well understood. IgG_4 antibodies act as blocking antibodies (better than IgG_1), but IgG_4 production may be also an epiphenomenon, its production reflecting conditions favorable for immune tolerance such as activation of T_{reg} cells, while regulatory B cells may produce IL-10 that promote IgG_4 production[177]. Very recent data complicate the opinion on the proven utility of such humoral biomarkers. In a randomized, double-blind placebo-controlled study using an allergen challenge chamber and quantitative, qualitative and functional analyses of allergen-specific IgE, IgG_{1-4} and IgA responses, clinical responders to grass pollen SLIT include both immunoreactive patients who exhibited strong increases in titers, affinity and/or blocking activity of grass-pollen-specific IgGs, as well as patients with no detectable antibody responses. Seric IgG responses may contribute to SLIT-induced clinical tolerance in some subjects, but additional immune mechanisms are involved in most patients[178]. Therefore, at the current level of knowledge, it is difficult to support the fact that antibody responses can be used as reliable biomarkers of AIT efficacy at an individual patient level.

Immune activation and immune tolerance soluble biomarkers

The immunopathogenesis of pollen respiratory allergy includes a preponderance of Th_2-type responses and the biochemical pathways triggered by Th_1-type cytokine interferon-*gamma*, such as tryptophan degradation by indoleamine 2,3-dioxygenase and neopterin production, might be altered[179]. Neopterin is a low molecular weight soluble biomarker of immune activation, synthesized from guanosine-triphosphate and produced preferentially by human monocytes/macrophages. Neopterin production and tryptophan catabolism through the kynurenine pathway, measured by the kynurenine-tryptophan ratio, are induced by interferon *gamma* (IFN-*gamma*), thus both are considered markers of T cell mediated immune activation. Serum neopterin concentrations can be determined by an enzyme immunoassay technique. SLIT may reduce serum neopterin levels, this phenomenon being possible due to the T_{reg} response able to induce IL-10 production, that may inhibit neopterin production. Thus, serum neopterin could be a serum biomarker of achieved immune tolerance toward the causal allergen in allergic patients successfully treated with SLIT[44,180]. Tryptophan and kynurenine serum concentrations seem to be higher in allergic rhinitis patients, especially out of pollen season. Simultaneous measurement of serum tryptophan and kynurenine may be performed by high performance liquid chromatography. Some authors suggested that non-responders to SCIT seem to have significantly higher tryptophan concentrations, higher tryptophan levels being a result of lower indoleamine 2,3-dioxygenase activity[179], and others revealed that serum tryptophan and kynurenine concentrations decrease after pollen SCIT, and a correlation between changes in tryptophan metabolism and neopterin concentrations was also possible after AIT[181].

The non-classical MHC class I molecule HLA-G plays important immunomodulatory activities. The differentiation of Tr_1 cells by tolerogenic IL-10-producing human DCs requires the IL-10-dependent ILT4/HLA-G pathway[182]. Leukocyte immunoglobulin-like receptor B2 (LILRB2) or ILT 4 (CD85d) is a human inhibitory immune receptor that recognizes HLA-G with a higher affinity[183]. Soluble HLA-G (sHLA-G) has increased serum values in patients with pollen allergic rhinitis studied outside the pollen season[184]. These can be determined by ELISA, while cell production of IFN-*gamma* is possible to be evaluated by enzyme-linked immunoabsorbent spot assay[185]. sHLA-G serum levels are reduced by pollen SLIT in allergic rhinitis patients and lowering of these levels and the increased IFN-*gamma* production after SLIT in pollen allergic rhinitis are significantly related phenomena. Thus, sHLA-G might be considered as a candidate biomarker of response to SLIT[43].

Apoptosis biomarkers

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)/Apo2L is a type Ⅱ transmembrane protein that was identified and cloned based on its sequence homology with members of the TNF ligand family. TRAIL-induced initiator caspase-8 and executioner caspase-3 cleavage is enhanced by IgE-dependent activation

of mast cells, which increases the expression of anti-apoptotic molecules FLIP (Fas-associated death domain-like IL-1 beta-converting enzyme-like inhibitory protease) and myeloid cell leukemia 1 (MCL-1 belonging to the bcl-2 family proteins), and a pro-apoptotic molecule Bcl-2 interacting mediator (BIM of cell death), thus fine modulating mast cell apoptosis[186]. Apoptosis of mast cells may be also regulated by some IgG receptors, such as $Fc_{gamma}RIIB$[187]. TRAIL is also present in cells, eosinophils, fibroblasts and airway epithelial cells. The soluble TRAIL (sTRAIL) is an apoptosis biomarker which can be measured in the serum by a sandwich enzyme-linked immunosorbent assay. sTRAIL levels may decrease after SCIT to healthy levels and may be of use as a marker of efficacy of immunotherapy in allergic rhinoconjunctivitis patients[41]. The role of sTRAIL in AIT is poorly understood and this makes the evaluation of the value of this biomarker difficult.

CONCLUSION

CRD biomarkers have proven utility in the assessment of sensitization to grass pollen allergens, allow the clinician to confirm genuine sensitization to the corresponding allergen plant sources and guide an accurate prescription of AIT, important in many regions of the world with great plant biodiversity and/or where pollen seasons may overlap. These disease-related molecular biomarkers, important tools for the future in allergy diagnostics, are hitherto available for the most important grass pollen allergens, although they have not currently replaced the classical existing methods of *in vivo*/*in vitro* allergy testing. Molecular diagnostic algorithms to guide pollen immunotherapy in some European regions are already designed[26].

It is difficult to estimate which of the presented candidate predictive biomarkers for grass pollen AIT will be validated in clinical practice, but those related to tolerogenic regulatory cellular responses are most promising. Some answers to questions regarding the upcoming guidelines for the use of predictive biomarkers for AIT and the possible role of combined application of biomarkers are not known and should be addressed as potential issues in future research. The search for candidate predictive biomarkers in AIT opens new opportunities for the early detection of clinical responders during AIT, for the follow-up of AIT patients and for the development of new allergy vaccines.

Molecular allergy biomarkers represent a complex area providing novel and relevant information for allergists and educational programs on their use in clinical practice are imperative[188].

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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AIM AND SCOPE

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REVIEW

Review of treatment assessment using DCE-MRI in breast cancer radiation therapy

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Abstract

As a noninvasive functional imaging technique, dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) is being used in oncology to measure properties of tumor microvascular structure and permeability. Studies have shown that parameters derived from certain pharmacokinetic models can be used as imaging biomarkers for tumor treatment response. The use of DCE-MRI for quantitative and objective assessment of radiation therapy has been explored in a variety of methods and tumor types. However, due to the complexity in imaging technology and divergent outcomes from different pharmacokinetic approaches, the method of using DCE-MRI in treatment assessment has yet to be standardized, especially for breast cancer. This article reviews the basic principles of breast DCE-MRI and recent studies using DCE-MRI in treatment assessment. Technical and clinical considerations are emphasized with specific attention to assessment of radiation treatment response.

Key words: Radiation treatment; Dynamic contrast-enhanced magnetic resonance imaging; Breast cancer; Treatment assessment

Core tip: Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) has shown great potentials not only in diagnosis, but also in therapy. DCE-MRI is a promising technique for assessing breast cancer radiation treatment due to its inherent sensitivity to the microvascular environment changes. Correlative studies have demonstrated proof concepts of DCE-MRI parameters as potential biomarkers. This article reviews the basic principles of breast DCE-MRI and recent studies using DCE-MRI in breast treatment assessment. Future clinical trials and research works are needed to develop standardized DCE-MRI assessment methods, towards the goal of individualized radiation therapy.

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INTRODUCTION

Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) is an advanced MRI technique that can be used to acquire tissue functional information noninvasively. Following the administration of low molecular weight contrast agent (CA), DCE-MRI is sensitive to microvessel density and vascular permeability differences that can be associated with tumor angiogenesis. Because of this merit, DCE-MRI has been investigated in various oncologic tasks including early diagnosis[1-5], tumor staging[6,7], treatment planning[8,9], and treatment response assessment[10-14]. To assess treatment response, the acquisition of pre-treatment DCE-MRI and post-treatment DCE-MRI scans are required to measure treatment induced changes[14,15]. The change could be quantitatively characterized by a few parameters, which can be derived

in the analysis of the DCE-MRI data. For intuitive comparison, simple semi-quantitative information can be obtained from the features of CA concentration evolution curve[16]. The quantitative functional information, such as micro-vascularity permeability, tissue perfusion and cellular density, must be obtained through the application of an appropriate pharmacokinetic model.

As a potential treatment assessment tool, DCE-MRI's application in breast cancer radiation treatment is of our particular interest. Currently, breast cancer is one of leading incidences in women. Earlier statistics shows one out of eight (12.5%) women will eventually be affected by breast cancer during her lifetime[17]. Since 1990, the death rate of breast cancer have steadily decreased in the United States due to earlier detection and improved treatment[18], and radiation therapy (RT) has become an important technique in breast cancer treatment. Currently, conserving treatment consisting of lumpectomy followed by 6 wk of daily external beam RT has become one of the common treatment regimes in United States. At the same time, some advanced radiation treatment techniques have been proposed to neutralize complexities in breast cancer treatment[19-21]. With its intrinsic superiority in soft tissue contrast and added ability of vascularity measurement, DCE-MRI is a particularly attractive technique in early assessment of breast cancer radiation treatment. The value of using DCE-MRI as a tool for breast cancer radiation treatment assessment relies on the accuracy of quantitative DCE-MRI parameters derived by modeling injected CA pharmacokinetics. However, this is far from straightforward[15]. Some DCE-MRI technical factors will potentially affect the consistency of measured parameters accuracy. For example, differences in pharmacokinetic parameters were observed using different temporal resolution and spatial resolution during image acquisition, and the effect of this tradeoff has yet to be clarified[22]. For clinical consideration, different pharmacokinetic models as well as the interpretation may lead to biased results[23]. Thus, optimizing and standardizing DCE-MRI measurement methods in breast cancer radiation treatment assessment presents as a prerequisite for its clinical application.

In this article, we outline the basic principles in breast DCE-MRI methodology and highlight some relevant techniques and theories in DCE-MRI application. We then present the current findings to date and discuss future directions for DCE-MRI in breast cancer radiation treatment assessment.

DCE-MRI MEASUREMENT AND ANALYSIS METHOD

Basic principles

DCE-MRI involves a sequential acquisition of magnetic resonance images of tissue before and after the intravenous injection of CA. The CA is usually a small molecular weight compound such as gadopentetate dimeglumine. T_2^* weighted MRI can be used right after the administration of CA in a few seconds to observe CA first-pass effect which contains perfusion information. Since the first-pass T_2^* effect is transient, the rapid imaging method performed over a single slice through tissue-of-interest (TOI) is necessary. This is of limited value in breast study because of the necessary larger volume coverage for comprehensive disease morphology assessment[24]. In contrast, T_1-weighted DCE-MRI technique is more commonly used in breast cancer research.

The T_1-weighted DCE-MRI is usually used over a longer time course in several minutes to measure the accumulation of low molecular T_1-shortening paramagnetic CA in the tissue. When CA enters into the tissue-of-interest, the tissue T_1 value decreases to an extent which is determined by the CA concentration. A CA concentration evolution curve as a function of time can be acquired from sequentially sampled T_1-weighted magnetic resonance images signal intensity at the TOI[25]. The CA concentration at each time point after the administration, $C(t)$, is calculated from longitudinal relaxation rate $R_1(t)$ [*i.e.,* the inverse of $T_1(t)$] and the longitudinal relaxation rate R_{10} before the CA administration with assumed linear dependence[26]:

$$R_1(t) = rC(t) + R_{10} \quad (1)$$

r is the longitudinal relaxivity of the CA at certain magnetic field strength. The conventional T_1 measurement methods are usually based on inversion recovery spin echo technique. This theory follows a spin inversion and waits for an inversion time TI before the data acquisition. Sometimes, multiple TIs are necessary to accurately estimate a wide range of T_1 values[27,28], which is the major contribution of long scan time. To reduce the scan time with uncompromised image quality, many T_1 scanning methods have been proposed in brain research domain[29-32]. Another T_1 mapping approach is to use multiple flip angles scans. To reduce imaging time, T_1 value can be obtained by simple dual flip angles technique[33]. In this method, the ratio of signals of two T1-weighted MR scans with different flip angle φ and ψ is expressed as P. With the general assumption about echo time TE < T_2^*, T_1 value can be calculated by equation (2):

$$f(\rho) = \frac{\rho sin\varphi cos\psi - cos\varphi sin\psi}{\rho sin\varphi - sin\psi}$$

$$T_1 = TR/ln[f(\rho)] \quad (2)$$

TR is denoted as repetition time. equation (2) is used for both $R_1(t)$ and R_{10} calculation. To get R_{10} information, two additional T1-weighted MR scans must be performed prior to DCE-MRI scan to get T_1 baseline information. These two scans with different flip angles are also called T1-calibrations. The following DCE-MRI scans are then acquired with flip angle φ (or ψ), and $R_1(t)$ is derived using the DCE-MRI signal at time point t and the T1-calibration with flip angle ψ (or φ) in equation (2). After applying longitudinal relaxation information into equation (1), the CA concentration evolution curve then can be expressed in pixel-by-pixel pattern or volume-of-

interest pattern.

For image acquisition, fast T1-weighted sequence is usually adopted for clinical studies. To cover the large breast imaging volume, the imaging time for each frame is relatively longer. Currently, the typical temporal resolution is about 1 min covering the whole breast with three-dimensional fast (3D) spoiled gradient echo (SPGR) dynamic sequence[34,35]. A recent feasibility study demonstrated that the temporal resolution could potentially be enhanced when compressed sensing theory was employed to reconstruct undersamped acquisitions[36].

Semi-quantitative analysis

Semi-quantitative analysis is usually performed on MR signal intensity-time curves or CA concentration evolution curves. In 1998, Daniel *et al*[37] proposed a patient classification scheme based on visually inspection on MR signal intensity-time curve shape. This scheme defines 5 types of curves. A change in the curve shape type to a higher number is considered as a transformation to a more aggressive type. For a more quantitative approach, the enhancement ratio (ER), which is the percent increase of MR signal intensity at the first acquirement after CA administration (also known as early contrast uptake ECU), is reported as a prediction of tissue physiological environment for routine clinical applications[38]. At the same time, some other quantities, such as initial wash-in rate, the wash-out rate, the maximum point, and extrapolation point were associated as important parameters for the description of curve shape. In the analysis of CA concentration evolution curve, the most frequently used parameter is the initial Area Under the Curve ($iAUC_\tau$). $iAUC_\tau$ denotes the integration of CA concentration evolution curve from injection point ($t = 0$) to a certain time point ($t = \tau$), and it parameterizes the initial rise of the evolution curve. The concept of onset time representing time lag between CA injection and the appearance of contrast in the tissue is also a commonly used biomarker. Similarly, the gradients of CA uptake and washout as well as the maximum concentration have been investigated in some studies[39,40]. Rigorous mathematic models were also introduced to describe the CA kinetic curve. For example, Fan *et al*[41] developed an empirical mathematical model (EMM) to parameterize the mathematical behavior of CA concentration evolution curve in transplanted rodent prostate tumors:

$$C(t) = A(1-e^{-at})^q \cdot e^{-\beta t} \cdot \frac{1+e^{-\gamma t}}{2} \quad (3)$$

A is the upper limit of CA concentration, a is the rate constant of CA uptake, β is the overall rate of CA washout, γ is the initial rate of CA washout, and q is the related to the radius of curvature of $C(t)$ at the transition from first-pass to initial washout. Results showed fitted parameters from EMM demonstrated the significant difference between metastatic tumors and nonmetastatic tumors. The same model was also demonstrated to be effective in differentiation of benign lesions from malignant lesions in a human breast study[42].

Quantitative analysis

In quantitative analysis, biological parameters depicting vascular permeability, tissue perfusion and extracellular volume fraction can be derived from CA concentration evolution curves by fitting into an appropriate pharmacokinetic model. For breast tissue, the most widely used pharmacokinetic model is the one proposed by Tofts *et al*[43] in 1991. This two-compartment model describes the bi-directional transendothelial movement of CA between blood plasma and the extravascular-extracellular space (EES) through capillary walls (Figure 1). There are three functional parameters in this model: K^{trans}, the transport rate of CA from blood plasma to EES; k_{ep}, the transport rate describing the return of CA from EES to blood plasma, and v_e, the volume fraction of EES in tissue. The three parameters are related by the equation $k_{ep} = K^{trans}/v_e$; as a result, K^{trans} and v_e were reported in most breast DCE-MRI studies. The measured CA concentration $C(t)$ consists of two components:

$$C(t) = C_{EES}(t) + v_p C_p(t) \quad (4)$$

In equation (4), $C_{EES}(t)$ is the CA concentration in EES, $C_p(t)$ is the CA concentration in blood plasma, and v_p is the plasma volume fraction in the tissue[44]. The $C_{EES}(t)$ term can also be expressed by the Kety Rate Law as the convolution of $C_p(t)$ with an exponential term[45]:

$$C_{EES}(t) = K^{trans} \int_0^t C_p(t') \exp[-\frac{K^{trans}}{v_e}(t-t')]dt' \quad (5)$$

K^{trans} is the CA extravasation rate, and v_e is the EES volume fraction. Tofts argued that plasma volume fraction v_p was very small for many TOIs including breast, so the contribution from $C_p(t)$ in equation (4) is neglected. Then equation (5) can be rewritten as equation (6), which is referred as Standard Tofts Model:

$$C(t) = K^{trans} \int_0^t C_p(t') \exp[-\frac{K^{trans}}{v_e}(t-t')]dt' \quad (6)$$

The knowledge of $C_p(t)$ is acquired separately from pharmacokinetic model and will be discussed later.

Though the Standard Tofts Model is acceptable in tumors with no large increase in blood volume, the assumption is likely to be invalid in some contexts as blood volume can increase markedly. As a result, some investigators incorporated the effects of possible significant vascular signals[46,47], and equation (6) is added by an additional vascular term:

$$C(t) = v_p C_p(t) + K^{trans} \int_0^t C_p(t') \exp[-\frac{K^{trans}}{v_e}(t-t')]dt' \quad (7)$$

The above equation is frequently called Extended Tofts Model. It was argued that Extended Tofts Model could be reliable in the region with higher vascular signal (abdomen, v_p up to 0.3) than the region with lower corresponding signal (brain, v_p up to 0.005)[48].

It has to be pointed out that both standard tofts

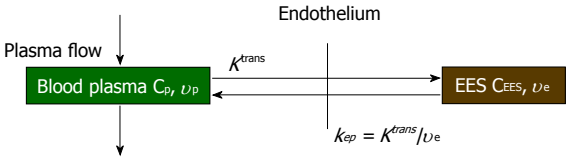

Figure 1 A sketch of two-compartment model. K^{trans}: Transport rate of CA from blood plasma to EES; k_{ep}: Transport rate of CA from EES to blood plasma; υ_e: Volume fraction of EES. The three quantities are related by $k_{ep} = K^{trans}/\upsilon_e$. EES: Extravascular-extracellular space.

model and extended tofts model are applied to the CA concentration evolution curve $C(t)$, which is oftenly obtained by equation (1). However, the linear dependence of CA concentration and longitudinal relaxation change is not always the case, because this statement is equivalent to assuming that interstitium behaves as a homogeneous solution. To use equation (1), the water exchange from the extravascular intracellular space (EIS) to the EES must be sufficiently fast; but in practice, this is not always guaranteed[49-51]. equation (1) is then modified by taking Bloch equations into account[52]:

$$R_1(t) = 1/2\,[R_{1i} + rC(t) + \frac{R_{10} - R_{1i} + 1/\tau_i}{v_e/f_w}] - 1/2[2/\tau_i - rC(t) - (\frac{R_{10} - R_{1i} + 1/\tau_i}{v_e/f_w})^2 + 4\frac{(1-v_e/f_w)v_e/f_w^{1/2}}{\tau_i^2}] \quad (8)$$

R_{1i} is the intracellular longitudinal relaxivity, r is the CA longitudinal relaxivity, $C_p(t)$ is the CA concentration in blood plasma, v_e is EES volume fraction, τ_i is the average intracellular water lifetime, and f_w is the fraction of water that is accessible to mobile CA. Since the Standard Tofts Model doesn't rely on the fast water change assumption, the $C(t)$ can be replaced by equation (6), leading to "Fast-Exchange Regime" FXR Model:

$$R_1(t) = 1/2\,[R_{1i} + rK^{trans}\int_0^t C_p(t')\exp[-\frac{K^{trans}}{v_e}(t-t')]dt' + \frac{R_{10} - R_{1i} + 1/\tau_i}{v_e/f_w}] - 1/2[2/\tau_i - rK^{trans}\int_0^t C_p(t')\exp[-\frac{K^{trans}}{v_e}(t-t')]dt' - (\frac{R_{10} - R_{1i} + 1/\tau_i}{v_e/f_w})^2 + 4\frac{(1-v_e/f_w)v_e/f_w^{1/2}}{\tau_i^2}] \quad (9)$$

In practice, R_{1i} is set to R_{10}, and f_w is assigned as a constant between 0 and 1. As seen in equation (9), a new variable τ_i is introduced in FXR Model. In theory, τ_i is the measurement of cell size. Presumably, as tumor cells apoptose in response to effective treatment, an decrease of τ_i would be observed. The utility of this parameter has yet to be fully studied[52].

Compared to the two-compartment models, the multi-compartment model has a potential capability of more precise description of pharmacokinetics inside human body. In a pilot study on mammary DCE-MRI, the tumor was modeled by 4 compartments and three of them were accessible to the CA from the central compartment (blood plasma). In addition, a peripheral compartment was introduced to distinguish normal tissues from the tumor[53]. Although the tumor heterogeneity was considered in this model, the in-tumor exchange pattern was still vague. As a nature of the multi-compartment model, the mathematic complexity limits the model's capacity in breast DCE-MRI study.

Aside from the conventional compartmental models, distributed-parameter (DP) models are seen as another category of DCE-MRI pharmacokinetic model. While the conventional compartmental models have been widely used for more than two decades, they may not possess sufficient realism CA concentration gradients within compartments are assumed to be zero; consequently, CA is assumed to distribute the compartments on arrival instantaneously[54]. On the contrary, DP models describe concentration gradients in vascular compartment as a function of both space and time. Several DP models have been proposed[55,56], but the application in breast clinical study is far from prevalent.

In all pharmacokinetic models mentioned above, the information of CA concentration in blood plasma at each time point $C_p(t)$, which is also known as Arterial Input Function (AIF), must be known prior to the model fitting. This knowledge can be achieved by imaging the major blood pool inside the field of view of images during DCE-MRI scan. For example, the study performed by Rijpkema *et al*[57] automatically extracted AIF data from DCE-MRI data in head-and-neck region tumor, prostate tumor and brain tumor cases. Unfortunately, such measurement is not feasible for clinical breast cancer studies because no large vasculature is qualified for MR sampling in breast tissue. Though Port *et al*[58] was able to acquire individual AIF through the visualization of aorta in the breast tumor study, the special procedure was not standardized in clinical protocols. Another approach is to use a population based AIF as an approximation of individual AIF[59,60]. A commonly used model is expressed by a bi-exponential decay[25]:

$$C_p(t) = D[a_1\exp(-m_1t) + a_2\exp(-m_2t)] \quad (10)$$

D is the CA administration dose as per unit of bodyweight. The two terms in this curve correspond to the fast dynamic equilibrium of CA between blood plasma and EES (represented by a_1 and m_1) and the slow renal removal of CA (represented by a_2 and m_2). Several groups of parameter values were reported[44,61].

As can be observed, appropriate AIF is important for accurate quantitative DCE-MRI analysis. However, current approaches in AIF analysis are far from satisfactions. Some investigators have made a lot of efforts in quantitative DCE-MRI analysis in absence of AIF knowledge. Inspired by positron emission tomography, Yankeelov *et al*[62] proposed a reference region model in 2005. This compartmental model compares the TOI's CA concentration evolution curve shape to that of a reference re-

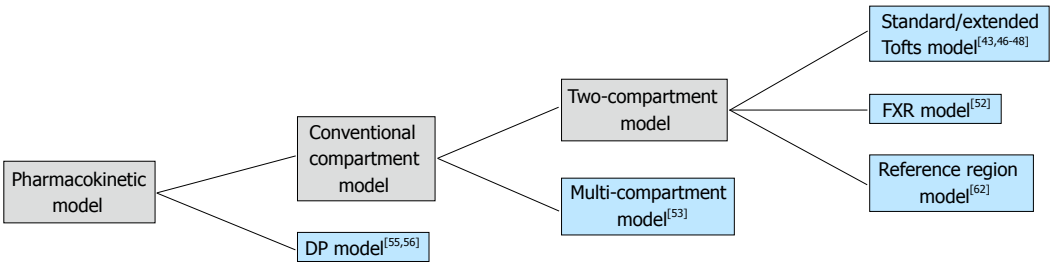

Figure 2 A hierarchical relationship diagram of the introduced pharmacokinetic models. DP: Distributed-parameter; FXR: Fast-exchange regime.

gion; as a result, the need of AIF information is eliminated. Based on two-compartment model, CA diffuses from blood plasma into EES of the reference region and the TOI respectively, and no exchange of CA exists between the reference region and the TOI. Following equation (1), the longitudinal relaxation signal for TOI, $R_{1,TOI}(t)$, can be derived from reference region's longitudinal relaxation signal $R_{1,Ref.Region}(t)$:

$$R_{1,TOI}(t) = r(R_{1,Ref.Region}(t) - R_{10,Ref.Region}) + r[(K^{trans,Ref.Region}/v_{e,Ref.Region}) - (K^{trans,TOI}/v_{e,TOI}) \cdot \int_0^t (R_{1,Ref.Region}(\tau) - R_{10,Ref.Region}) \cdot \exp(-K^{trans,TOI}/v_{e,TOI}(t-\tau))d\tau] + R_{10,TOI} \quad (11)$$

As can be seen, the $K^{trans,TOI}$ and $v_{e,TOI}$ must be known in the reference region model. In the mouse tumor study, these values were assigned to the muscle values from publications. But evidently, individual variation of K^{trans} and v_e values of the selected reference region may result in errors in the values of TOI.

The aforementioned pharmacokinetic models are organized in a hierarchal scheme in Figure 2. To get the functional parameters, appropriate model fitting algorithm must be applied to the DCE-MRI data. For clinical application, the mathematical fitting method should to be fast and accurate. Currently, non-linear Levenberg-Marquart algorithm have been widely used in DCE-MRI studies[63]. Some other fitting methods also have been investigated[64,65]. In some cases, however, the convergence of the fitting algorithm is not guaranteed, thus the accuracy of model fitting may be compromised. Schmid *et al*[61] raised a semi-parametric approach with which the AIF is convolved with a set of B-splines to produce a design matrix from Bayesian penalized spline models (*P*-spline). The model parameter is then obtained from the deconvolved response function. At a cost of computation time, the semi-parametric technique was suggested to be more accurate when traditional fitting methods were poor during *in vivo* validation.

DISCUSSIONS ON DCE-MRI IMPLEMENTATION AND ANALYSIS IN RADIATION TREATMENT ASSESSMENT

Biologically optimized radiotherapy is a novel technique in which a treatment plan is tailored individually to emphasize variations of pathological context[66]. This approach is made possible by the assessment of treatment response, an indispensable tool in the evaluation of new treatment techniques. As a non-invasive approach, the conventional medical images, including X-ray, ultrasound, computed tomography (CT) and MRI, have been used to evaluate the radiation treatment through the tumor morphological assessment[67]. However, this approach may be of limited value in gauging the radiation treatment efficacy because the tumor may have already developed its radiation resistance when the observation of morphology change is available[68]. In addition, the population-based evaluation standard in patient's follow-up care after the radiation treatment may not be optimal considering the pathological variations among individuals. The functional analysis of cancer treatment with the possible individualized standards may be a promising approach. The reliability and validity of the functional assessment has been proved in some pilot studies[69,70]. In the radiation treatment context, the non-invasive functional imaging during the early stage of the fractionated therapy would be promising in providing early evidences in treatment management. The unnecessary systemic toxicity and the treatment delays could be avoided as treatment plans could be optimized based on individualized pathological analysis during the treatment regime[71].

The non-invasive function imaging approach is also valuable in the development of advanced treatment techniques. The recent progress in breast cancer radiation treatment allows the accurate delivery of a high dose in one or several fractions. Due to the unconventional dose size and fraction scheme, the biological response of the new techniques should be fully investigated in view of safety and effectiveness. One of the factors of radiation response is tumor oxygenation. Radiobiology theory claims that hypoxia leads to decreased radiation damage induced cell death with an increased level of DNA repair enzymes and radial scavengers[68]. Hypoxia can also cause genome changes which favor the radiation resistant cell population, thus promoting the development of cells with more aggressive phenotypes[66,72-74]. The varying degree of hypoxia is characterized by microvasculature abnormalities, including abnormal microvessel architectures and an increased permeability[75]. Due to the natural sensitivity of the microvascular environment, DCE-MRI measurement parameters were studied in correlation with

Table 1 Dynamic contrast-enhanced magnetic resonance imaging parameter correlations with physiological parameters at breast tissue

Ref.	Correlation		Direction of correlation	*P*
	Physiological parameter	DCE-MRI measurement		
Buadu *et al*[76], 1996	MVD	Amplitude	+	< 0.01
		Slope maximum	+	< 0.01
		Enhancement Maximum time	-	< 0.01
		Tumor size	..	NS
Stomper *et al*[77], 1996	DNA S-phase percentage	Signal enhancement amplitude	..	NS
		Signal enhancement rate	..	NS
		Signal washout	..	NS
Hulka *et al*[78], 1997	Tumor grade	RSI	+	< 0.01
Matsubayashi *et al*[79], 2000	Peripheral-central MVD ratio	Early rim enhancement	+	0.048
	Peripheral-central fibrosis ratio	Early rim enhancement	-	< 0.01
	Central-peripheral fibrosis ratio	Delayed rim	+	0.013
Gianfelice *et al*[80], 2003	Residue tumor percentage after MRIgFUS	ISI	+	NR
		MDF	+	NR
		PEI	+	NR
Su *et al*[81], 2003	VEGF	Apparent Vb	..	NS
		In-flux rate and distribution volume in interstitial space (VeK1)	..	NS
		Out-flux rate (K2)	..	NS

NS: Not significant; NR: Not reported; RSI: Relative signal intensity; MVD: Microvessel density; ISI: Increase in signal intensity; MDF: Maximum difference function; PEI: Positive enhancement interal; Vb: Vascular volume; VEGF: Vascular endothelial growth factor.

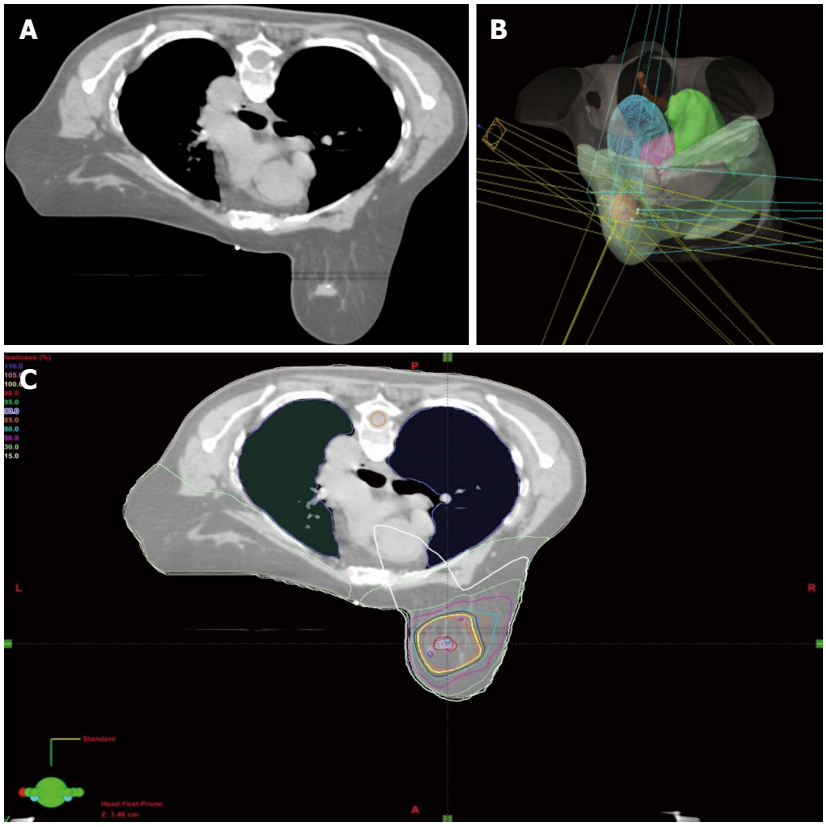

Figure 3 Radiation treatment planning. A: A computed tomography simulation image for a selected patient breast stereotactic body radiosurgery (SBRT) treatment plan; B: 3D planned beams view for the selected patient's SBRT plan; C: Calculated conformal dose distribution of the selected patient's SBRT plan.

physiological variables at the breast site. Some of the results are listed in Table 1. Although histopathological studies have shown discrepancies in the outcome, the results suggest that DCE-MRI is suitable for RT assessment of perfusion, permeability and oxygenation[66].

Conventionally, the workflow of RT consists of CT simulation, radiotherapy planning and treatment delivery. Specifically, a breast cancer patient may be scanned with CT simulator to obtain the CT data for treatment planning, as shown in Figure 3A. Based on the CT data, a state-of-the-art RT plan can be developed with a conformal dose distribution, as shown in Figure 3B and 3C. The conventional workflow is summarized in Figure 4A for an easy appreciation. The conventional workflow may, however, miss one critical stage of treatment: treatment assessment. Effective treatment assessment would

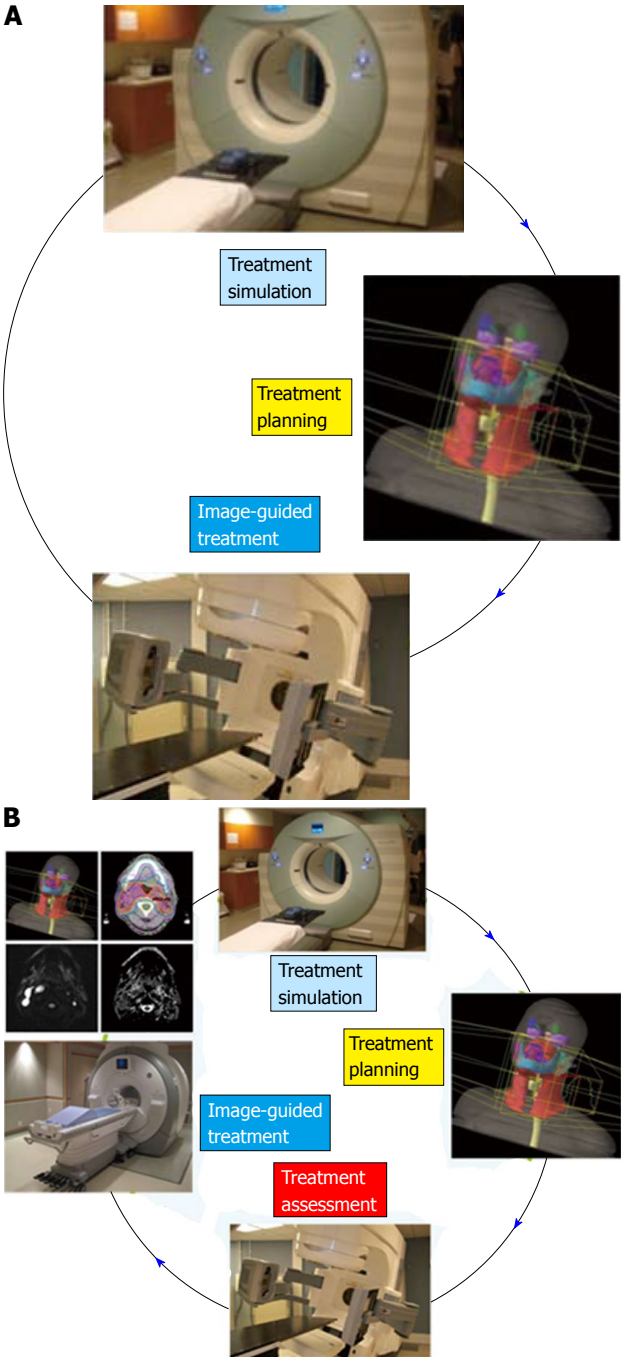

Figure 4 Conventional radiation treatment workflow. The proposed workflow (A) with treatment assessment component (B). Radiation treatment assessment can be used in plan optimization based on understanding towards biological response.

not only potentially help optimize the radiation treatment strategy, but also could provide valuable insights on the future development of RT. As shown in Figure 4B, the proposed workflow of RT consists of four critical components: CT simulation, radiotherapy planning, treatment delivery and treatment assessment. To assess treatment response using DCE-MRI, one DCE-MRI scan must be obtained before the treatment for baseline data. In addition to standard CT image, the pre-treatment DCE-MRI scan can also be used in target delineation during treatment planning. After radiation treatment, at least one

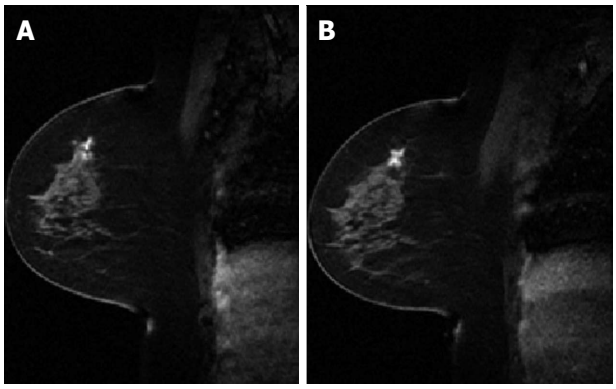

Figure 5 A comparison between pre-treatment dynamic contrast-enhanced magnetic resonance imaging image (A) and post-treatment dynamic contrast-enhanced magnetic resonance imaging image (B).

post-treatment DCE-MRI scan must be acquired, and the DCE-MRI parameters derived by the semi-quantitative analysis and/or the quantitative analysis will be compared to the corresponding baseline values. Multiple post-treatment scans allow the longitudinal study of biological response through the parameter dynamic change. Figure 5 illustrates an example of pre-treatment and post-treatment DCE-MRI image comparison from a selected patient. Table 2 lists some studies of the DCE-MRI application in breast cancer radiation treatment assessment. Currently, limited studies have been done in this specific area; further study focusing on breast radiation response assessment is desirable and urgent. In addition to the radiation treatment assessment studies, some representative breast non-radiation treatment studies are also included to provide valuable references and insights on the DCE-MRI application in radiation treatment assessment.

Although DCE-MRI is a promising and a powerful tool for assessing treatment response, there are several technical factors to be considered during its clinical implementation, which are crucial to the precise meaning of the derived results. Some key points in DCE-MRI analysis will be briefly discussed below to provide some valuable references for the future work with specific interest on DCE-MRI radiation treatment assessment.

T_1 measurement uncertainty

Of all available fast T_1 measurement techniques, SPGR imaging with dual flip angles has the superiority in noise efficiency compared with others[30,87]. For high precision T_1 measurement which is often necessary in brain studies, multiple flip angle pairs can be adopted to minimize the statistical uncertainty of measured values. In the breast region, acceptable accuracy in T_1 value can be achieved if the optimized flip angle pair is found. These two optimal angles are obtained by minimizing T_1 variance which comes from the manipulation of the error propagation theory on equation (2) above[33]. In another pilot study of Deoni *et al*[30], it was argued that optimization of T_1 accuracy can be achieved when the product of normalized dynamic range and the fractional signal is maximized. Both methods yield similar optimal flip angle pairs for a certain

Table 2 Dynamic contrast-enhanced magnetic resonance imaging studies in breast cancer treatment assessment

Ref.	Treatment method	DCE-MRI measurement	Major results
Dao *et al*[82] 1993	Conserving radiation therapy	Signal intensity	The signal intensity over time in localized fibrosis showed a different pattern from the one in tumor recurrence; early increased signal intensity of the lesion within 3 min after CA administration were observed in recurrent tumors
Drew *et al*[83] 2001	Neoadjuvant chemoradiotherapy	DCE-MRI image	DCE-MRI detected the presence of residual disease with 100% accuracy
Hayes *et al*[16] 2002	Neoadjuvant chemotherapy	K^{trans}	The absolute change in the K^{trans} values correlated negatively with the pretreatment values. The trend is more obvious for responding patients
Martincich *et al*[38] 2004	Primary chemotherapy	ECU	ECU reduction was associated with a MHR (OR = 2.50, 95%CI: 0.263-23.775); no statistical significance was observed (P = 0.42)
Overmoyer *et al*[84] 2004	Antiangiogenic drug	K_{ep}	Correlative studies suggested a reduction of tumor K_{ep} and tumor MVD
Wedam *et al*[85] 2006	Antiangiogenic drug	K^{trans} and v_e	K^{trans} decreased by 34.4% (P = 0.003), and v_e was decreased by 14.3% (P = 0.002) after the treatment using bevacizumab
Yankeelov *et al*[52] 2007	Neoadjuvant chemotherapy	K^{trans}, v_e, and τ_i (average intracellular water lifetime)	Significant (P < 0.05) changes were seen in K^{trans} and v_e; K^{trans} in voxels with values in 0.2-0.5 min^{-1} before treatment were found decreased (P < 0.035)
Chang *et al*[86] 2013	SBRT	$iAUC_{5min}$	The mean $iAUC_{5min}$ in GTV increased from 1.93 ± 0.20 mmol-min to 2.31 ± 0.16 mmol-min, about 16% (P = 0.012) after treatment

Highlighted studies were related to radiation treatment. DCE-MRI: Dynamic contrast-enhanced magnetic resonance imaging; ECU: Early contrast uptake; MHR: Major histopathological response; GTV: Gross tumor volume; SBRT: Stereotactic body radiotherapy.

TR and T_1 range. In a simulation study of breast pharmacokinetic parameter estimation[88], both K^{trans} and v_e were proved to be overestimated if tissue T_1 is underestimated; on the contrary, if the T_1 is overestimated, K^{trans} and v_e were less severely underestimated. Specifically, when the ductal native tissue T_1 value is underestimated by 65%, K^{trans} would be potentially overestimated by 531%. With the same T_1 underestimation, v_e hit its ceiling threshold with any combination of true K^{trans} and v_e. As can be seen, optimization of T_1 measurement in the DCE-MRI imaging protocol is crucial for accurate quantitative pharmacokinetic analysis.

B1 inhomogeneity effect

The error in the optimization of the nominal flip angle is a consequence of B1 inhomogeneity which becomes more prominent at higher magnetic field strength. Kuhl *et al*[89] proved that for the same breast lesion, the enhancement rate obtained at 3.0 T magnetic field was lower than the respective rate at 1.5 T magnetic field. In the further study[90], B1 field across the bilateral breast MRI field at 3.0 T showed substantial variation, and the variation was independent of coil type. The actual pulse angle varied between 22° and 12.5° over the field of view. Similarly, up to 55% error of nominal flip angle was observed from the healthy volunteer's B1 maps at 3.0 T[91]. In a breast DCE-MRI study at 3.0 T[92], the median measured B1 field at the right side of breast (in prone position) was reduced by nearly 40% of the expected value. Experiment and simulation showed that a reduced B1 field decreased the ER of dynamic signal curve, and this trend became more prominent when CA uptake was higher. The pharmacokinetic parameters were also affected by B1 inhomogeneity through the varying flip angle[88]. Simulation results showed when the flip angle was underestimated by 55% of its nominal value, K^{trans} measurement dropped by 66%, and 55% overestimation of flip angle led to 61% increase of K^{trans}. As v_e increased, K^{trans} sensitivity to the varying flip angle was strengthened. On the other hand, v_e showed similar changing pattern except the sensitivity to varying flip angle was independent of K^{trans} value. In contrast to 3.0 T, the B1inhomogeneity is less prominent at 1.5 T and is less studied.

Temporal resolution

The temporal resolution in DCE-MRI is directly dependent on the imaging volume. In a clinically feasible scan which is a part of treatment planning imaging, the frame time covering the whole breast is about 1 min[52]. Theoretically, the reduced temporal resolution would affect the precision of pharmacokinetic analysis by changing the CA concentration evolution curve. In an animal study with 4.7 T magnetic field strength[93], DCE-MRI data was first acquired with 5 s temporal resolution. The data was then downsampled to temporal resolutions ranging from 15 to 85 s. The CA concentration curve showed large discrepancies during the earlier phase. Quantitatively, as temporal resolution decreased, K^{trans} was progressively underestimated from 4% to 25%, and v_e was overestimated from 1% to 10%. In another simulation study[88], as temporal resolution reduced, K^{trans} underestimation was more pronounced at higher nominal values, while v_e displayed a 2% minor variation.

One simple way to increase the temporal resolution is to image the lesion only. For radiation treatment assessment purpose, the planning target volume (PTV) is a good candidate, but the knowledge of the TOI must be known prior to scan. As an alternative strategy, undersampling the image with intensive mathematic operation can also increase the temporal resolution[36]. However, since there is no gold standard of true values for pharmacokinetic parameters, the benefit of high temporal

resolution imaging is limited. Nevertheless, improvement of high temporal resolution in DCE-MRI will be a continuing interest for researchers.

Importance of AIF

Ideally, the AIF should be measured from DCE-MRI data for each case, as it varies between individuals in reflection of cardiac output, vascular tone and renal function[15]. Unfortunately, as discussed above, the measurement is not practical in clinical routine imaging because no larger vascularity is within field of view. Besides, the measurement demands high temporal resolution which is not achievable in whole breast imaging[22,59,94,95]. The idealized mathematical model functions are commonly used, though the used functions make no attempt to reflect the true blood supply to the volume of interest[22]. Some other quantitative methods require no AIF information[62,96], but further studies must be done focusing on human breast tissue. In conclusion, one should be aware that AIF methodology leads to potential inaccuracy of pharmacokinetic parameters.

Pharmacokinetic model

There is no uniform standard of choosing a pharmacokinetic model in quantitative DCE-MRI analysis. The current consensus is that simple models describing the CA transfer rate from the blood plasma to the EES (K^{trans}) and the EES volume fraction (v_e) should be used for the assessment of vascularity change[15]. The Standard Tofts model and the Extended Tofts model have been widely used due to their simplicity. Despite the limitation of describing the biological picture of CA transport, the two simple models have been proved to be very useful with limited temporal resolution and without accurate AIF information[94]. However, these two models are not identical: in a comparative study into the robustness of compartmental modeling on abdominal tumors and gliomas, the K^{trans} calculated by Extended Tofts Model was considerably lower than the value from Standard Tofts Model, while v_e maintained similar range in both methods[48].

In pursuit of a more realistic biological mechanism, other models have been evaluated the aspect of accurate parameter reproducibility. For example, initial application of the FXR Model suggested that K^{trans} and v_e were underestimated by values up to 300% in the assumption of a linear relationship between CA concentration and longitudinal relaxivity change[50,51]. The FXR model was also reported as with the most complete statistical description of DCE-MRI time courses for the patients selected in the study[97]. As a DP model, adiabatic approximation of the tissue homogeneity model (ATH) was proved to be more effective in CA dynamic curve fitting than Tofts models for Time-resolved angiography With Stochastic Trajectories data[98]. However, these comparisons cannot be seen as the evidence of superiority in biological reality. The pharmacokinetic parameter, for example, K^{trans}, does not absolutely measure capillary permeability in any model, though it is often assumed to do so; the exact meaning depends on the specific model used for analysis. For instance, the reduction of K^{trans} can be interpred as a reduction of blood permeability in ATH model, or a reduction of both blood flow and permeability in Tofts models. As a result, the choice of model reflects the tradeoff between parameters that are either simple in math but lack biological specificity or more physiologically congruent but less stable in math.

Region of interest and statistical analysis

Radiation treatment has certain regions of interest including Gross Tumor Volume, Clinical Target Volume and PTV. Data analysis performed over the TOI using the average CA concentration or average signal intensity generates the regional parameters. Though this method is faster, it ignores heterogeneity within the volume of interest. Alternatively, the parameters can be extracted in a pixel-by-pixel pattern within the TOI. The statistics summary such as the mean or median value and standard deviation can be used for assessment[52,90,100]; this method can describe the parameter distributions and limited information about microvessel heterogeneity[15]. In practice, the second method may be too slow for clinical application depending on voxel number and mathematic complexity of model fitting. The challenge can be neutralized by selecting meaningful voxels through certain simple metric[101], or using the advanced GPU acceleration to reduce analysis time[102-105].

CONCLUSION

DCE-MRI is a promising technique for assessing breast cancer radiation treatment due to its inherent sensitivity to change in the microvessel environment. Correlative studies have demonstrated proof concept of DCE-MRI parameters as potential biomarkers. Presently, an insufficient number of clinical studies have been done in breast cancer radiation treatment. Currently, progress has been achieved in pharmacokinetic model development in pursuit of precise physiology description; however, these methodologies have yet to be fully studied in correlation with clinical outcome in breast cancer radiation treatment. For future work, the study of new pharmacokinetic model with special interests on breast tumor pathology will help improve the interpretation of the DCE-MRI parameter. Advancement in DCE-MRI image acquisition with high spatial and temporal resolution will contribute to the utility of DCE-MRI application in radiation treatment assessment. On the perspective clinical trials are needed with primary aim designed to test standardized DCE-MRI assessment methods for both image acquisition and quantitative biomarker derivation. This is a crucial step towards the goal of individualized radiation treatment planning.

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REVIEW

MicroRNAs in lung cancer

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Abstract

MicroRNAs have become recognized as key players in the development of cancer. They are a family of small non-coding RNAs that can negatively regulate the expression of cancer-related genes by sequence-selective targeting of mRNAs, leading to either mRNA degradation or translational repression. Lung cancer is the leading cause of cancer-related death worldwide with a substantially low survival rate. MicroRNAs have been confirmed to play roles in lung cancer development, epithelial-mesenchymal transition and response to therapy. They are also being studied for their future use as diagnostic and prognostic biomarkers and as potential therapeutic targets. In this review we focus on the role of dysregulated microRNA expression in lung tumorigenesis. We also discuss the role of microRNAs in therapeutic resistance and as biomarkers. We further look into the progress made and challenges remaining in using microRNAs for therapy in lung cancer.

Key words: Lung cancer; MicroRNA; MiRNA

Core tip: Lung cancer is a prolific and high mortality disease, with few effective treatments. MicroRNAs have a role in the biogenesis and maintenance of lung cancer, with oncogenic and tumor suppressive effects. They are also a significant factor in resistance to current forms of therapy. There is evidence that microRNAs will be useful as diagnostic and predictive biomarkers in the future and, if delivery challenges can be overcome, they may become integrated into treatments.

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INTRODUCTION

MicroRNAs (miRNAs) are small (19-22 nucleotides) non-coding RNAs that were first discovered in *Caenorhabditis elegans*[1]. MiRNAs silence their target genes by binding to the 3' untranslated region (3'-UTR) of target messenger RNAs (mRNAs), causing either degradation or inhibition of translation. In animals, miRNAs are part of an approximately 70-100 nucleotides RNA with a stem-loop structure, known as a pre-miRNA that is included in hundreds or thousands of nucleotides long primary miRNA precursors (pri-miRNAs). The first step of microRNA biogenesis involves the transcription of the pri-miRNA and this is mediated by RNA polymerase Ⅱ (Pol-Ⅱ)[2], although a minor group of microRNAs can be transcribed by RNA polymerase Ⅲ (Pol-Ⅲ)[3]. Then the pri-miRNA is processed in the nucleus by the RNase Ⅲ enzyme Drosha and the protein Pasha/DGCR8 into pre-miRNAs[4]. The pre-miRNA undergoes a second processing step within the cytoplasm, and a small double-stranded RNA struc-

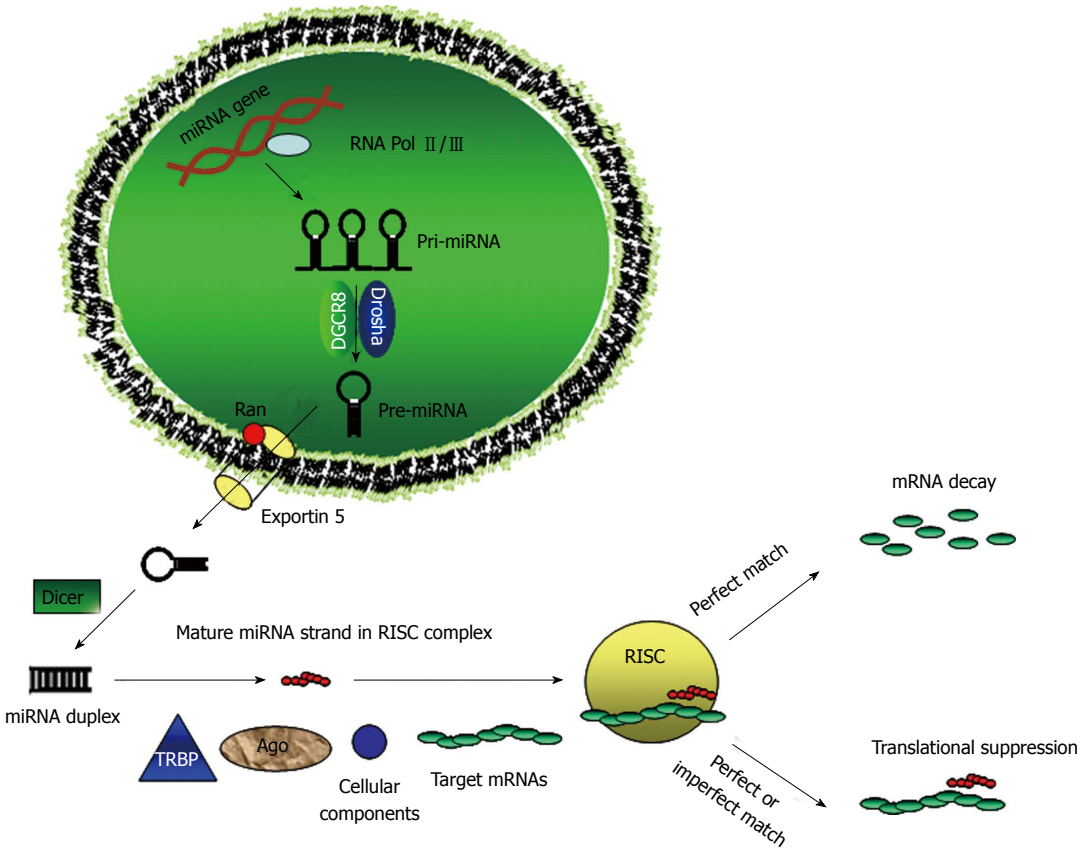

Figure 1 MicroRNA biogenesis. RISC: RNA-induced silencing complex; TRBP: HIV-1 transactivating response RNA-binding protein.

ture approximately 22 nucleotides in length is excised from the pre-miRNA hairpin by another RNase Ⅲ enzyme, Dicer[5,6]. Finally, the mature single-stranded microRNA is loaded into the RNA-induced silencing complex, which mediates the degradation or translation inhibition of target mRNA by binding to its seed sequence in the target mRNA's 3'-UTR (Figure 1). Dysfunctional microRNAs are commonly found in a variety of solid cancers and are attractive candidates for next-generation therapeutics.

Lung cancer remains the leading cause of cancer-related death worldwide, and non-small cell lung cancer (NSCLC) accounts for approximately 80% of all cases[7,8]. Although novel therapies targeting early diagnosis have been developed, the 5-year survival rate for NSCLC patients remains at a low 15%[9]. Takamizawa *et al*[10] were the first to relate microRNA expression to lung cancer. Since then there have been large number of studies relating microRNA expression with lung cancer. Here we describe the roles of microRNAs as tumor suppressors and oncogenes and their role in prognosis and diagnosis of lung cancer. Moreover, we discuss the contribution of microRNAs in radioresistance and chemoresistance as well as several therapeutic ventures involving microRNAs in lung cancer.

MICRORNAS AS TUMOR SUPPRESSORS AND ONCOGENES

Numerous studies have reported finding mutation or aberrant expression of microRNAs in lung cancer patients. Investigators have shown that microRNAs whose expression is altered in tumors, may function as a novel class of oncogenes or tumor inhibitor genes. Several microRNAs are dysregulated in lung cancer, target cancer-relevant targets and have been documented to have tumor-suppressing or tumor-promoting activity in *in vitro* and/or *in vivo* models in lung cancer.

TUMOR SUPPRESSOR MICRORNAS

Let-7

Let-7 was the first microRNA found to be dysregulated in lung cancer. Indeed, Takamizawa *et al*[10] reported that let-7 expression levels are frequently reduced in lung cancers both *in vitro* and *in vivo*. Let-7 subsequently was reported to be inversely correlated with RAS protein expression in lung cancer tissues, providing a possible mechanism for let-7 in lung cancer[11]. Kumar *et al*[12] used both inducible and constitutive expression systems to show substantial tumor suppression by let-7g in xenografts and a mouse lung tumor model in a K-Ras dependent manner. Enforced expression of let-7a in A549 cells decreased NIRF (Np95/ICBP90-like RING finger protein) leading to a coordinated increase in $p21^{WAF1}$[13]. NIRF binds with higher affinity to the methylated CpGs of the promoter region through its SRA (SET and RING finger associated) domain and possibly recruits histone deacytylase-1 (HDAC1) through the same domain. This

recruitment of HDAC1 to the methylated promoter regions of some tumor suppressor genes such as $p21^{WAF1}$ can suppress their expression[14]. Recently[15], let-7c was observed to be inversely correlated to and directly target ITGB3 (integrin b3, also known as CD61)[16], and MAP4K3, a member of the MAP4K family[17] in NSCLC tissues. These observations support the assumption that let-7 may act as a tumor suppressor microRNA.

miR-126

MiR-126 overexpression in lung cancer cell lines decreases Crk protein and leads to decreased adhesion, migration and invasion[18]. Crk is an adaptor protein that mediates several intracellular signal pathways[19] that are important in cell growth, motility, differentiation, and adhesion[20]. Liu *et al*[21] used an RNA protection assay to show downregulation of miR-126 in many lung cancer cell lines. MiR-126 overexpression efficiently reduced the expression of vascular endothelial growth factor (VEGF) and inhibited cell proliferation *in vitro* and tumorigenicity *in vivo*. Futhermore, enforced expression of miR-126 impaired NSCLC cell proliferation and tumor growth in xenografts model by targeting PIK3R2 and thus regulating the PI3K-Akt pathway[22], confirming a tumor suppressive role in lung cancer.

miR-145

Introduction of miR-145 was reported to dramatically suppress the c-Myc/eIF4E pathway by targeting c-Myc, which has been demonstrated to be crucial for cell proliferation in NSCLC cells. Cell growth was inhibited and the G_1/S transition was blocked by miR-145 overexpression in A549 and H23 cells[23]. Enforced expression of miR-145 negatively regulated the expression of EGFR and NUDT1[24]. NUDTI (8-oxo-dGTPase) is involved in accumulated mis-incorporation of oxidized 8-oxo-dGTP into DNA that can lead to cell dysfunction and death[25,26]. When miR-145 was overexpressed in A549 cell line there was a reduction in proliferation of $CD133^+$ lung adenocarcinoma-initiating cells and tumorosphere growth capacity. This tumor suppressive effect involved miR-145 targeting of octamer-binding protein 4, a transcription factor of embryonic stem cells[27,28].

miR-200

miR-200c plays a central role in the process of epithelial-mesenchymal transition (EMT) in highly invasive/aggressive NSCLC cells by targeting TCF8 (ZEB1) thus restoring its regulatory target E-cadherin[29,30]. Loss of miR-200c in invasive cells was observed to be a result of hypermethylation of the promoter region[30]. Studies by Yang *et al*[31] reveal a novel Jagged2/miR-200-dependent pathway that mediates lung adenocarcinoma EMT and metastasis in mice. They showed that Jagged2 increased the expression of GATA-binding factors that in turn suppressed members of miR-200 family driving EMT and reciprocally, miR-200 inhibited GATA3 expression reversing EMT. Furthermore, overexpression of miR-200 in murine lung adenocarcinoma cells decreased their growth and metastasis by targeting Flt1/VEGFR1[32] confirming the EMT suppressive function of miR-200.

miR-34

The miR-34 family (miR-34a, -34b and -34c) is directly regulated by p53 and has been reported to induce apoptosis and cell cycle arrest in cancer cells[33,34] and is being studied for its anti-tumorigenic nature. The receptor tyrosine kinase Axl protein induces proliferation, migration and invasion in cancer[35]. Mudduluru *et al*[36,37] found an inverse correlation between Axl and miR-34a in NSCLC cell lines. ZEB1, a transcriptional repressor that promotes metastasis by downregulating microRNAs like the miR-200 family[38], drives prometastic actin cytoskeletal remodeling in NSCLC cells by inhibiting miR-34a expression[39]. Exogenous miR-34 prevented tumor initiation and progression in a therapeutically resistant $Kras^{G12D/+}$; $Trp53^{R172H/+}$ mouse lung cancer model[40]. Studies in our lab have shown that miR-34a and miR-34c overexpression increased TNF-related apoptosis inducing ligand (TRAIL)-induced apoptosis and decreased invasiveness of lung cancer cells by targeting PDGFR-α and PDGFR-β[41].

ONCOGENIC MIRNAS

miR-17-92

The miR-17-92 intronic cluster comprising seven different microRNAs namely miR-17-5p, -17-3p, -18a, -19a, -19b-1, -20a, and -92 was found by Hayashita *et al*[42] in lung cancer, mostly in small cell lung cancer (SCLC). Antisense oligonucleotides against mir-17-5p and miR-20a induced apoptosis in miR-17-92 overexpressing lung cancer cells[43]. Several targets have been studied for the various members of the miR-17-92 family. MiR-17-92 directly targeted hypoxia-inducible factor (HIF)-1A and overexpression of c-Myc led to downregulation of HIF-1A and induction of miR-17-92, suggesting that the induction of miR-17-92 may play a part in c-Myc–mediated repression of HIF-1A[44]. MiR-17-92 counterbalanced the generation of DNA damage in RB-inactivated SCLC cells by reducing γ-H2AX foci[45]. Matrix metalloproteinase (MMP) play an essential role in tumorigenesis by regulating migration and invasion of cells. *In vivo* MMP activity is controlled by the balance between MMPs and inhibitory proteins such as Reversion-inducing Cysteine-rich protein with Kazal motifs (RECK)[46]. STAT3 was shown to upregulate miR-92a thereby repressing RECK *via* post-transcriptional inhibition and thus promoting MMP activity[47]. These studies suggest that miR-17-92 may be an excellent therapeutic target candidate in the treatment of lung cancer.

miR-21

miR-21 has been reported to be overexpressed in nine types of solid tumors including lung[48] as well as in hematological malignancies such as leukemia[49,50] and has great therapeutic potential for lung cancer. Over-expression

Table 1 MicroRNAs in resistance

miRNAs	Target	Drug/ Treatment	R/S	Ref.
miR-9, let-7g	NFκB1	Radiotherapy	S	[66]
miR-34b	BCL2	Radiotherapy	S	[67]
miR-214	N/A; PTEN	Radiotherapy; gefitinib	R	[68,88]
miR-155	FOXO3A; Apaf-1	Radiotherapy; cisplatin	R	[69,78]
miR-210	Stabilizes HIF-1A in normoxia	Radiotherapy	R	[70]
miR-181	N/A	Cisplatin	S	[72]
miR-451	N/A	Cisplatin	S	[73]
miR-98	TP53	Cisplatin	R	[74]
miR-497	BCL2	Multiple drugs	S	[75]
miR-200b	BCL2, XIAP; E2F3	Cisplatin; docetaxel	S	[76,86]
miR-21	PTEN, BCL2	Cisplatin	R	[79]
miR-135a	APC	Paclitaxel	R	[82]
miR-100	Plk1	Docetaxel	S	[85]
miR-337-3p	STAT3, RAP1A	Paclitaxel and docetaxel	S	[87]
miR-221, miR-222	P27kip1	TRAIL	R	[91]
miR-130a	MET	TRAIL	S	[92]
miR-212	PED	TRAIL	S	[93]

R: Resistance; S: Sensitivity; XIAP: X-linked inhibitor of apoptosis protein; STAT3: Signal transducer and activator of transcription 3; RAP1A: Ras-related protein Rap-1A; MET: met proto-oncogene (hepatocyte growth factor receptor); PED: Astrocytic phosphoprotein PEA-15; FOXO3A: Forkhead box O3; N/A: Not available; p27kip1: Cyclin-dependent kinase inhibitor 1B.

of miR-21 enhanced tumorigenesis through inhibition of negative regulators of the Ras/ MEK/ERK pathway and inhibition of apoptosis[51]. MiR-21 was observed to repress phosphatase and tensin homolog (PTEN) and stimulated growth and invasion in NSCLC cell lines[52]. PTEN overexpression mimicked the same effects of anti-miR-21 such as inhibiting migration and invasion in NSCLC cells[53]. MiR-21 was shown to directly target the 3'-UTR of human mutS homolog 2, a core DNA mismatch repair (MMR) protein[54], thus affecting the cell cycle and cell proliferation in NSCLC cell lines[55] further underlining the oncogenic role of miR-21 in lung cancer.

miR-31

MicroRNAs and DNA mismatch repair have been linked to human cancer progression. Human mutL homolog 1 (*hMLH1*) is a core *MMR* gene and reduced expression of hMLH1 can lead to genetic instability in NSCLC[56]. MiR-31-5p was reported to directly target and to be inversely correlated with MLH1 expression in NSCLC cell lines. Knockdown of miR-31-5p increased hMLH1 protein expression and induced a cell cycle arrest at G_2/M phase in NSCLC cells[57]. MiR-31 was overexpressed in malignant lung tissues from humans and targeted the tumor-suppressive genes large tumor suppressor 2 (LATS2) and PP2A regulatory subunit B alpha isoform (PPP2R2A). Engineered knockdown of miR-31 repressed lung cancer cell growth and tumorigenicity in a dose-dependent manner[58]. These findings reveal that miR-31 acts as an oncogenic miRNA in lung cancer by targeting specific tumor suppressors for repression.

miR-221 and -222

Our group has shown that miR-221 and -222 directly targeted PTEN and Tissue inhibitor of metalloproteinase 3 tumor suppressors inducing TRAIL resistance and enhancing cellular migration through the activation of the AKT pathway and MMPs. We observed that MET oncogene activates miR-221/222 through the c-Jun transcription factor[59]. The p53 up-regulated modulator of apoptosis (PUMA) suppresses growth of A549 cells through induction of apoptosis and sensitizes cells to chemotherapeutic agents and irradiation[60]. It was reported that miR-221/222 directly target and co-modulate PUMA expression and knockdown of miR-221/222 in A549 cells inhibited cell proliferation and induced mitochondrial-mediated apoptosis[61]. Thus, targeting miR-221/222 could be an effective strategy for therapy in lung cancer.

MICRORNAS IN RESISTANCE

Radiotherapy, usually in combination with chemotherapy, is routinely used in lung cancer treatment, especially for NSCLC, allowing for better local control of the disease and reduction of metastasis occurrence. Both radiation resistance and chemoresistance is common, preventing successful long-term therapy and contributing to the dismal prognosis. Investigators are constantly trying to develop new effective therapies by studying the mechanisms behind resistance. Aberrant expression of several miRNAs has been correlated with the development and progression of tumors, and the reversal of their expression has been shown to modulate the cancer phenotype, suggesting the potential of miRNAs as targets for anti-cancer drugs. Here we describe the putative role(s) of microRNAs in the development of resistance to therapy (Table 1).

Radiotherapy resistance

When living cells are exposed to ionizing radiation (IR), a series of alterations occurs including transformation, cell cycle distress, mutations, sister-chromatid exchanges, chromosome aberrations, DNA repair, and apoptosis[62,63]. Among the IR-responsive genes, the activation of NFκB1 following genotoxic stress allows DNA damage repair and cell survival[64] and its inhibition can increase sensitivity of cancer cells to chemotherapeutic agents and radiation exposure[65]. Overexpression of miR-9 has been shown to down-regulate the level of NFκB1 in γ-irradiated H1299 human lung cancer cell line and decrease the surviving fraction of γ-irradiated cells. Interestingly, let-7g also suppressed the expression of NFκB1, although there is no canonical target site for let-7g in the NFκB1 3'-UTR[66]. Tumor suppressor p53 is another key player of the complex DNA damage response activated in response to IR[63]. Overexpression of p53-regulated miR-34b[33,34] in p53 wild type A549 cells increased radiosensitivity at low doses of radiation and this effect was not observed in p53 null H1299 cells[67].

Several microRNAs are involved in inducing resistance to irradiation. MiR-214 was shown to be upregulated

in radioresistant NSCLC cells relative to radiosensitive counterparts and its overexpression protected radiosensitive cells against RT-induced apoptosis[68]. Incubation of NSCLC cell lines in hypoxic environments was reported to induce miR-155 expression and decrease its target, FOXO3A, a tumor suppressive transcription factor that regulates cell cycle and apoptosis. These increased levels of miR-155 radioprotected lung cancer cells and vice versa[69]. In another study, enforced expression of miR-210 increased radioresistance of NSCLC cells by stabilizing HIF-1A[70]. These studies reveal a therapeutically important link between miRNA expression, hypoxia, and irradiation.

Chemotherapy resistance

Platinum agents like cisplatin and carboplatin are some of the principal chemotherapeutic agents used for treatment of NSCLC. These agents induce their cytotoxic effects by targeting cellular DNA and are active against a number of tumour types[71]. However numerous studies have shown that an initial success associated with partial responses or disease stabilization is followed by the selection of chemotherapy-resistant tumor cells, leading to chemotherapeutic failure. Numerous microRNAs have been implicated in cisplatin resistance. Galluzi and colleagues reported miR-181a and miR-630 to be the most upregulated miRNAs after cisplatin (CDDP) treatment however, pre-miR-181a enhanced while pre-miR-630 reduced CDDP-triggered cell death in A549 cells by modulating steps of the intrinsic pathway of apoptosis[72]. Another group observed that ectopic expression of miR-451 might be involved in sensitizing A549 cells to cisplatin by inducing apoptosis *via* inactivation of Akt signaling pathway and enhancement of caspase-3 activity[73]. Zhang *et al*[74] showed that transfection with miR-98/miR-453 inhibited p53 expression and upon treatment with cisplatin, the expression of miR-98 decreases, while p53 increases. This led them to speculate that regulation of p53 pathway might play an important role in the action of cisplatin on A549 cell growth. Separate studies by Zhu *et al*[75,76] on miR-497 and miR-200b/429 cluster in multidrug resistant A549/CDDP cell line indicated an increased sensitivity to cisplatin in part by modulation of apoptosis *via* targeting only B-cell CLL/lymphoma 2 (BCL2) or both BCL2 and X-linked inhibitor of apoptosis, respectively. In the cytosol, Apaf-1 can bind with cytochrome-c released from the mitochondrial inter-membrane, and activate the initiator caspase-9, eventually resulting in cellular apoptosis[77]. MiR-155 was observed to be inversely correlated to Apaf-1 in lung cancer tissues. Silencing miR-155 or overexpressing Apaf-1 in A549 cell lines greatly increased the sensitivity of A549 cells to cisplatin treatment through an Apaf-1 mediated pathway, involving increased expression of Bax and caspase-9[78]. MiR-21 was reported to be critical in platinum resistance in NSCLC and modulated the sensitivity of NSCLC cells to platinum, at least in part, by regulating PTEN and BCL-2 expressions[79].

Taxanes, such as paclitaxel and docetaxel, are chemotherapeutic drugs that stabilize microtubules and inhibit their disassembly to tubulin interfering with proper formation of the mitotic spindle, which leads to activation of the mitotic spindle checkpoint and mitotic arrest[80]. Drug-treated cells then undergo apoptosis as a result of the abnormal mitosis[81]. Studies reporting the role of microRNAs in taxane resistance can provide novel adjuvant strategies along with taxanes in the treatment of lung cancer. Knockdown of miR-135a was reported to upregulate adenomatous polyposis coli gene (*APC*) and sensitize paclitaxel-resistant NSCLC cell lines to paclitaxel-induced cell death[82]. APC is a tumor suppressor that regulates the mitotic checkpoint by binding to microtubules during mitosis[83]. Polo-like kinase (Plk)-1 is a cell cycle protein that plays an important role in spindle dynamics and chromosome segregation during mitosis[84]. Feng *et al*[85] showed that introduction of miR-100 resensitized docetaxel resistant SPC-A1/DTX cells to docetaxel by suppression of cell proliferation, enhancement of apoptosis, and cell arrest in G_2/M phase of cell cycle at least partially by Plk-1 targeting. The same group also reported that ectopic expression of miR-200b reversed docetaxel resistance of SPC-A1/DTX cells in part by targeting E2F3[86]. Du and colleagues identified a novel regulatory pathway involving STAT3 and RAP1A that modulates miR-337-3p mediated paclitaxel sensitivity in lung cancer cells[87].

Patients with NSCLC who have activating epidermal growth factor receptor (EGFR) mutations derive clinical benefit from treatment with EGFR-tyrosine kinase inhibitors (EGFR-TKIs)-namely gefitinib and erlotinib. However, these patients eventually develop resistance to EGFR-TKIs. Wang *et al*[88] established a gefitinib resistant cell line-HCC827/GR and found that miR-214 was significantly up-regulated in these cells compared to control HCC827 cells. Knockdown of miR-214 in HCC827/GR resulted in upregulation of PTEN and inactivation of p-AKT and this in turn re-sensitized the cells to gefitinib. To understand the role of microRNAs in TKI-resistant NSCLCs, our group examined miRNA dysregulation mediated by TK receptors. MiR-30b, -30c, -221 and -222 were found to be modulated by both EGFR and MET receptors whereas miR-103 and miR-203 were controlled only by MET. We showed that these miRNAs influenced the response to gefitinib of NSCLC cells *in vitro* and *in vivo* by inhibiting the expression of the genes encoding BCL2-like 11 (BIM), apoptotic peptidase activating factor 1 (APAF-1), protein kinase Cε (PKC-ε) and sarcoma viral oncogene homolog[89].

Treatment with TRAIL induces programmed cell death in a wide range of transformed cells, both *in vitro* and *in vivo*, without producing significant effects in normal cells[90]. However, a significant proportion of human cancer cells are resistant to TRAIL-induced apoptosis, and the mechanisms of sensitization vary among cell types. To define novel pathways that regulate TRAIL-sensitivity in NSCLC, our lab performed genome-wide expression profiling of microRNAs. Levels of miR-221 and -222 were increased in TRAIL-resistant NSCLC cells and their knockdown rendered CALU-1-resistant cells sensitive to TRAIL. Conversely, H460-sensitive cells

Table 2 MicroRNA in diagnosis and prognosis. Traditional methods of diagnosing and evaluating lung cancer and their corresponding potential microRNA biomarkers

	Traditional Procedures	Possible microRNA biomarkers
Diagnosis		
Detect abnormalities	X-ray, CT scan	21
Confirm malignancy	Biopsy, sputum/fluid cytology	let-7, 29a, 34c, 205, 375
Prognosis		
Staging	CT scan, PET, MRI	21, 125b, 155, 182/183
Mutational status	Sequencing, PCR, microarray	21, 155

CT: Computed tomography; PET: Positron emission tomography; PCR: Polymerase chain reaction; MRI: Magnetic resonance imaging.

treated with pre-miR-221 and -222 developed a resistance. Interference with TRAIL signaling by miR-221 and -222 was mainly through targeting p27kip1[91]. Another study from our lab further reported that miR-130a, expressed at low level in lung cancer cell lines, by targeting MET was able to reduce TRAIL resistance in NSCLC cells through the c-Jun-mediated downregulation of miR-221 and miR-222[92]. Ectopic expression of miR-212 increased TRAIL-induced cell death in NSCLC cells by targeting PED/PEA-15 (PED), a death effector domain family member with a broad anti-apoptotic function[93]. These studies enhance our understanding of the mechanisms responsible for TRAIL resistance.

MIRNAS IN DIAGNOSIS

The diagnosis of lung cancer is performed through several methods with varying degrees of sensitivity and reliability. X-ray imaging, along with positron emission tomography and computed tomography (CT) scans, is often the first diagnostic procedure utilized. While these methods provide valuable information when anomalies are easily visible, problems with lung segmentation and positioning in the chest cavity, human error and competent detection software prevent imaging from always producing successful diagnoses. Similarly, while tissue sampling though bronchoscopy has become the standard practice in diagnosing lung cancer, it presents its own difficulties, including complications in obtaining viable samples due to patient symptoms, proper imaging and tumor position[94].

MicroRNAs show potential as biomarkers for the diagnosis of lung cancer that can complement and improve upon other techniques. Promising lung cancer microRNA biomarkers can be found circulating in the bloodstream, in sputum and inside cells, and are detected at an abnormal level when cancer is present. Ideally, these biomarkers should be detected through minimally invasive methods and with limited discomfort to patients. There are currently several dozen microRNAs under investigation for their biomarker properties (Tables 2 and 3).

MiR-21 has a well-documented correlation to lung cancer. Detected in both serum and sputum, elevated miR-21 corresponds to lowered survival rate, lymphoid invasions and KRAS mutations[53,95]. Promisingly, assays for miR-21 in sputum from lung cancer patients have shown higher sensitivity than traditional sputum cytology with very high specificity[96,97]. MiR-21, in combination with miR-210 and miR-486-5p, was shown to be expressed significantly higher in the plasma of patients with malignant solitary pulmonary nodules (SPNs) compared to those with benign SPNs. Solitary pulmonary nodules have been increasingly diagnosed with the improvement of CT scan technology and its widespread use. However, only a small fraction of SPNs are malignant. The combination of miR-21 testing and CT scans could provide a minimally invasive method of determining the cancer status of patients with SPNs[98].

Table 3 MicroRNAs with potential relevance to common types of lung cancer treatment

Treatment	Potential prognostic biomarkers	Potential role in resistance
Surgery	Let-7, 21	
Radiotherapy	155, 210	Let-7g, 9, 34, 155, 210, 214
Chemotherapy	21, 125b	21, 30b/c, 98, 100, 103, 130a, 135a, 155, 181, 200b, 203, 212, 214, 221/222, 337, 451, 453, 494, 630

MiR-155 is a prominent oncomiR, with various roles in lung cancer including proliferation and drug resistance. Used in a panel with miR-197 and miR-182, miR-155 was able to distinguish between NSCLC patients and control samples by real time PCR of plasma. Patients with metastasizing cancer consistently exhibited higher levels of plasma miR-155, which could additionally aid in staging the disease[99]. Several studies have found that miR-155 is only elevated in EGFR/KRAS-negative lung cancer. Samples from surgically resected lung specimens and fine needle aspirations (FNAs) both demonstrated this effect[100,101]. FNAs are considered to be safe, minor surgical procedures compared to excisional biopsies, thus further development of its use for collection of miR-155 to determine mutational status could be beneficial. An even less invasive technique was used by Yao and colleagues to determine levels of miR-155 *in vivo*[102]. They developed a novel molecular beacon that can be introduced into mice with lipid-DNA complexes and detect miR-155 in lung cancer xenografts through *in vivo* fluorescent imaging. The authors posit that these results may be translatable to human lung tumors, possibly improving on the problematic imaging resources currently available for diagnostics.

The miR-183 family (miR-96, miR-182, and miR-183) is a group of oncomiRs that have been confirmed to be overexpressed in lung tumors and serum in NSCLC. Targets for these miRs support a variety of biological processes, including growth, migration, invasion and angiogenesis. MiR-182 in particular has been found to be strongly correlated to primary tumors while all three are expressed more in squamous cell carcinoma than in ad-

enocarcinoma[103-105]. Additionally, miR-182 showed a high specificity and sensitivity, and readily differentiates stage I lung cancer from normal control samples, making it a tantalizing possibility for non-invasive clinical diagnostics. MiR-183, on the other hand, has been demonstrated as being able to differentiate between early and late stage NSCLC, while not being able to discriminate early stage lung cancer from normal cells[106].

The miR-34 family, in particular miR-34a and miR-34c, has been shown by multiple groups to be potential biomarkers in lung cancer[100]. Mascaux and colleagues detailed an inverse relationship between miR-34 levels and lung carcinogenesis, and later expounded on this by showing that changes in lung cell histology are reflected by miR-34c independent of any treatment[107]. However, these studies used biopsies as their source tissue, and have not yet confirmed that other extraction methods (sputum, serum, *etc*.) could be used to circumvent normal tissue sampling and histology. Akbas *et al*[108] have found that dysregulation of 34c can be confirmed through serum in chronic obstructive pulmonary disease (COPD) - an inflammatory disease that increases the risk of lung cancer - making further development of 34c as a lung cancer biomarker a likely avenue of research.

NSCLC is the most common form of lung cancer, and is divided into two subtypes, squamous cell carcinoma (SCC) and adenocarcinoma. Diagnosing the correct subtype is critical for treatment and microRNA biomarkers that are able to distinguish between these subtypes would be a useful tool in a clinical setting. MiR-205 - a tumor suppressor - has been found by several researchers to be a highly effective identifier of squamous cell histology through its downregulation, both in NSCLC tissues and serum[109,110]. Similarly, members of the let-7 family are significantly downregulated in SCC, likely due to the fact that let-7 regulates RAS expression, and RAS mutations are far more common in SCC than adenocarcinomas[111,112]. As lung tissue has one of the highest expressions of let-7 in the body, its characteristic decrease in SCC has the potential to make it an easily identifiable biomarker[113]. However, because this large decrease in expression has only been identified in lung tissue and not sputum, serum or bronchial fluids, the only current options for assaying let-7 are tissue biopsy or bronchial brushing. Thus, let-7 as a biomarker will have to show greater efficacy than traditional cytopathology to warrant clinical use.

Though less common and with comparatively little research, small cell lung cancer presents serious problems for patients, with a tendency toward rapid and widespread metastasis. Therefore, accurate and expeditious diagnostic markers are desirable. Two studies have found miR-375 to accurately discriminate between NSCLC and SCLC. Huang *et al*[114] used snap-frozen and paraffin-embedded surgical lung specimens, finding miR-29a and miR-375 to be superior to traditional cytopathology for diagnosing SCLC. In the other study, Zhao and colleagues found extremely elevated miR-375 expression in four human SCLC cell lines and four SCLC-like cell lines generated in mice[115]. These results are promising, but require more study with larger sample populations and examinations of extracellular microRNA levels to evaluate the usefulness of miR-375 as a clinical biomarker.

MIRNAS IN PROGNOSIS

An essential facet of cancer treatment involves the correct and efficient prognosis of the type of cancer and the expectations of survival and mortality. This prevents the unneeded use of potentially harmful drugs, and allows for the correct prescription of treatment strength and severity. As microRNAs have been confirmed to play roles in lung cancer development, migration and response to therapy, they may also find future use as biomarkers to give accurate prognoses to physicians.

Liu *et al*[116] found that miR-21 was significantly elevated in the serum of NSCLC patients with lower survival rates and showed a strong association with lymph node metastasis and advanced clinical stage. Yang *et al*[117] confirmed this result with a meta-analysis while others found similar results in three ethnically-diverse cohorts, including significant associations between elevated miR-21 and high-mortality stage I tumors[118,119]. These findings have the potential of allowing physicians to quickly evaluate and escalate treatments in response to early stage NSCLC diagnoses. Studies examining post-operative lung cancer patients also found that miR-21 serum levels significantly decreased in response to successful surgery, with higher miR-21 expression corresponding to shorter survival time and disease recurrence[120]. However, another study evaluating the use of miR-21 as a predictive biomarker in SCLC found no correlation between miR-21 expression and patient outcome[121]. The same study found similar results with 6 other important NSCLC-related miRs, underlying both the inherent differences between SCLC and NSCLC and also the paucity of data involving SCLC biomarkers. MiR-155 has been shown to have a similar elevation in expression in NSCLC, which is associated with low survival and high rates of recurrence[122,123]. Both miR-21 and miR-155 have been examined in sputum samples and found in readily detectable quantities, and while Xie and collaborators found only miR-21 produces adequate differentiation in expression for use as a biomarker[97], others have found using both in combination with three other miRs to be a highly sensitive panel for clinical applications[124].

The let-7 family, in addition to discriminating between SCC and adenocarcinoma, has also been found to be associated with survival rate. Low let-7a expression has been shown by multiple studies to correlate to a poor prognosis, both pre- and post-operative, particularly in SCC[125]. There is some evidence that this preferential prognostic ability comes from the squamous cell carcinoma's reliance on the downregulation of tumor suppressor miRs, including let-7, compared to adeocarcimoma's dependence on the upregulation of oncomiRs[111].

Several miRs have been identified as having the potential to predict the effectiveness of therapy. MiR-125b is an oncomiR that has been found to be significantly increased in stage Ⅲ and Ⅳ NSCLC. Cui *et al*[126] examined the expression of a panel of miRs, finding that miR-125b levels were markedly higher in patients that did not respond to cisplatin treatment. This corroborates several other studies that found that miR-125b inhibited cisplatin-induced apoptosis in breast and ovarian cancers[127,128]. Similarly, miR-21 shows promise as predictive biomarker for the response to adjuvant platinum based chemotherapies (cisplatin, oxaliplatin, *etc*.) in NSCLC[79]. Serum taken from patients after surgery and platinum based treatment showed elevated levels of miR-21 compared to a pre-treatment baseline if there was a low chemotherapeutic response. A recent study found that serum miR-210 consistently determined the success of platinum based chemotherapy. MiR-210 is upregulated in NSCLC and recent findings have shown that patients who responded well to treatment had significantly lower expression of miR-210 in serum, near levels expected in healthy control subjects[129]. Another study reported that NSCLC cells overexpressing miR-210 were conferred with radioresistance as well, displaying an ability to rapidly repair double-strand DNA breaks[70]. As radiotherapy is a common treatment in lung cancer, with more than half of patients receiving irradiation, potential microRNA biomarkers - miR-210 for example - that predict the efficacy of this procedure would make an immediate impact on patients and physicians' decisions. In its role as a mediator of radioresistance, miR-155 may also have potential as a prognostic biomarker, with elevated expression corresponding to lower survival rate in patients who have received radiotherapy[69,130].

MIRNAS AS POTENTIAL TREATMENTS

Increasing evidence supporting the essential role of microRNAs in the machinery of cancer points to the possibility of using microRNAs as treatments in lung cancer. The most evident problem blocking clinical use of microRNA therapies is delivery. Specifically targeting cancer cells, maintaining microRNA stability in bodily fluids and penetration of cellular membranes are areas of intense investigation. Some modifications to microRNA and anti-microRNA oligonucleotides (AMOs), including 2'-O-methyl, 2'-O-methoxyethyl and locked nucleic acids, provide nuclease resistance for greater longevity during serum transport, but additional methods are needed to enhance the cell permeability of these molecules[131].

Liposomes are a promising avenue of microRNA therapy delivery. These artificial, spherical vesicles made from a lipid bilayer are used to administer pharmaceutical drugs, microRNA or small interfering RNA (siRNA). Experiments using mouse models have found both neutral lipid emulsions and cationic lipoplexes to be effective in delivering microRNAs to lung tumors. Multiple studies have used a neutral lipid emulsion to deliver tumor suppressors miR-34a and let-7 to NSCLC tumors in mice, which resulted in a 60% reduction in tumor area[132,133]. Wu and colleagues found that cationic lipoplexes were over 50-fold more effective in delivering pre-miR-133b, a known inhibitor of NSCLC proliferation, to NSCLC mice than NeoFX complexes, a standard transfection reagent, and with lower cytotoxicity[134,135]. Recently, this same team used cationic lipoplexes to deliver miR-29b into murine A549 xenografts, finding similar success in cellular penetration, along with documenting a decrease in tumorigenicity and improved functionality of cisplatin[136]. Shi and colleagues have used a novel technology - solid lipid nanoparticles (SLNs) - to transport AMOs to suppress miR-21 in lung cancer and introduce miR-34a into lung cancer stem cells, inhibiting cell migration and inducing cell apoptosis[137]. SLNs boast superior cellular uptake rates and decreased oligonucleotide degradation, which allow AMOs to be introduced without stability-adding modifications that reduce specificity. Future directions for liposome therapy reseach include increasing stability of liposomes and better targeting through the use of tumor-recognizing antibodies and peptides. Some studies have already shown that incorporating ligands that target overexpressed lung cancer receptors into liposomes dramatically improves liposome uptake into NSCLC cells[138] while others have used synthetic antigens to activate tumor-targeting immune cells[139].

Viral delivery systems are a platform that offers naturally high infection rates and high miRNA expression levels for lung cancer treatment. Adenoviruses have been used as a vector for the delivery of miR-122, a tumor suppressor, into NSCLC NCI-H460 cells. The resulting 2000-fold higher expression of miR-122 led to the activation of intrinsic apoptotic pathways[140]. Sun and colleagues used a lentiviral vector to infect hepatocellular carcinoma cells with osteopontin-suppressing microRNAs that decreased tumorigenicity in mice and down-regulated the oncogenic MEK/ERK/1/2 pathway[141]. These results may be transferable to lung cancer, as osteopontin has been identified as a pro-metastatic factor in NSCLC[142]. Overall, adenoviruses are considered the better option for microRNA vectors as they do not integrate into the genome. There are complications with viral delivery of microRNAs, though, including immunogenicity and cellular toxicity that will need to be addressed in further research.

As liposome delivery often produces toxicity and requires considerable optimization to maintain adequate stability and efficacy, and viral vectors are limited by immunogenicity much research has recently focused on the use of nanoparticles. These small, solid spheres offer reduced immune response, lower toxicity and cheap, efficient production methods that result in high complex stability.

Protamine, a biologically derived molecule, has been complexed with microRNA, resulting in higher transfection rates than with lipoplexes[143]. Chen *et al*[144] utilized protamine complexes with miR-34a to inhibit the growth of lung metastases of melanoma. These nanoparticles incorporated a liposome shell around the nanoparticles,

with great effectiveness. Further studies are needed to determine the necessity of liposomal encirclement of protamine complexes, taking into account microRNA degradation, cellular uptake and immune response. Gold and silica nanoparticles have also been utilized in microRNA delivery[145,146], but as of yet, there are no studies demonstrating their use in treating lung cancer.

CONCLUSION

MiRNAs have become recognized as key players in cancer. Their ability to regulate expression of cancer-related genes has immense implications for the diagnosis and treatment of cancer. Lung cancer is the leading cause of cancer-related death worldwide and currently has a substantially lower survival rate than many other common cancers. In this review, we discuss how dysregulated miRNA expression has been shown to contribute to the genesis and maintenance of lung cancer, through the down-regulation of tumor suppressors and up-regulation of oncomiRs. Additionally, miRNAs may be essential in the development of chemo- and radioresistance in lung cancer. Due to their importance in the regulatory structure of cancer, miRNAs may soon be used to improve diagnosis and predictions of outcomes and response to therapy, although more studies will be needed with larger sample groups to resolve conflicting reports of disease-state expression patterns for some miRNAs. Implementing miRNAs and anti-miRNAs as treatments presents some additional difficulties, mostly related to delivery and stability inside the body, but holds promise as a less toxic therapy that can target multiple genes simultaneously. The investigation into miRNAs and cancer is still relatively new, and more study will be needed to form consensuses on the critical functions of miRNAs inside cancer cells, what information can be gleaned from changes in their expression and the best methods for therapeutic administration, but these unique compounds show great promise as tools against lung cancer.

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REVIEW

Novel agents and new therapeutic approaches for treatment of multiple myeloma

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Abstract

This review summarizes the therapeutic strategies and the drugs actually in development for the management of myeloma patients. Multiple myeloma is caused by the expansion of monoclonal plasma cells and secretion of M-protein (immunoglobulins, Bence Jones protein and free light chains). Multiple myeloma still remains an incurable disease with a high incidence rate in the elderly, despite the introduction of several new therapeutic agents (bortezomib, lenalidomide and thalidomide) which have changed its natural history. The high heterogeneity of this disease leads to large differences in clinical responses to treatments. Thus, the choice of the best treatment is a difficult issue. However, the introduction of new drugs has made it possible to achieve high response rates and good quality responses with long-term disease control. Interactions between tumor cells and their bone marrow microenvironment play a pivotal role in the development, maintenance, and progression of myeloma, inducing also drug resistance. These knowledges have improved treatment options, leading to the approval of new drugs which not only target the malignant cell itself, but also its microenvironment. These agents are in preclinical/early clinical evaluation and they appear to further improve disease control, but their use is still not approved outside of clinical trials.

Key words: Immunomodulators; Multiple myeloma; New drugs; Proteasome inhibitors; Target therapy

Core tip: The aim of this review is to summarize and point out the current therapeutic strategies and the drugs actually in development for the management of multiple myeloma. The rationale of the new treatment strategies is found in their efficacy in targeting tumor cells and their microenvironment. Our understanding of multiple myeloma (MM) pathogenesis including the intracellular mechanisms as well as the interactions between MM cells and their microenvironment has helped the discovery of several targets that have become the focus of drug development. The goal is to improve patient's survival and to control the disease in a long-term fashion, maintaining the quality of life of our patients.

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INTRODUCTION

The treatment of multiple myeloma (MM) is rapidly

evolving (historical progress in MM treatment options is shown in Figure 1). Near the old active classes of drugs including alkylators (*e.g.*, melphalan and cyclophosphamide), corticosteroids (*e.g.*, prednisone and dexamethasone), and anthracyclines (*e.g.*, doxorubicin), new drug formulations (*e.g.*, liposomal doxorubicin) and new active classes of drugs such as proteasome inhibitors (*e.g.*, bortezomib) and immunomodulatory drugs (*e.g.*, thalidomide and lenalidomide) have been introduced in myeloma therapy[1].

Changes in treatment strategies due to the introduction of novel drugs have been able to significantly improve the quality of responses. In fact, if in the past, complete remission (CR) in MM was rare to achieve, while the introduction of new treatments has increased the rate in younger patients as well as in the non-transplant setting. CR represents a surrogate marker of long survival. It correlates with the long-term progression-free survival (PFS) and overall survival (OS). Achieving CR and sustaining CR within a 3-year landmark from the treatment initiation were associated with highly superior survival. Actually, we agree that "the more profund the remission is, the longer the duration of response is"[2].

In this paper we review the novel agents that could shape future directions for MM management. As far as possible treatment should be individualized. Also, it should be recognized that it is not necessarily best practice to refer particular therapies at specified time points. In the future, it is likely that MM therapy will be "risk-adapted" and the presence or absence of specific prognostic factors may determine the choice of therapy both at diagnosis and relapse.

THERAPY OF MM

Improvements in MM biology knowledge have led changes into the rationale of modern therapy[3]. In fact, the role of microenvironment in myeloma pathogenesis and progression has been well established[4] and the new drugs are designed to target myeloma plasma cells and their microenvironment simultaneously. This different approach has changed the natural history of MM, which still remains an incurable disease, but the survival is significantly improved.

NEWLY DIAGNOSED MM

Initial therapy for MM depends on the eligibility for autologous stem cell transplant (ASCT) (younger fit patients). Patients who are considered potential candidates for ASCT receive 2-4 cycles of a non-melphalan-containing regimen and then proceed to stem cell harvest[5]. After stem cell harvest, most patients move on to ASCT. However, depending on the response to initial therapy and patient's preference, initial therapy can be resumed after stem cell harvest, delaying ASCT until first relapse. The role of early *vs* delayed ASCT is an argument of debate[6]. On the contrary, the second ASCT in patients who do not achieve almost a very good partial response (VGPR) after the first transplant seems to be the best option[7].

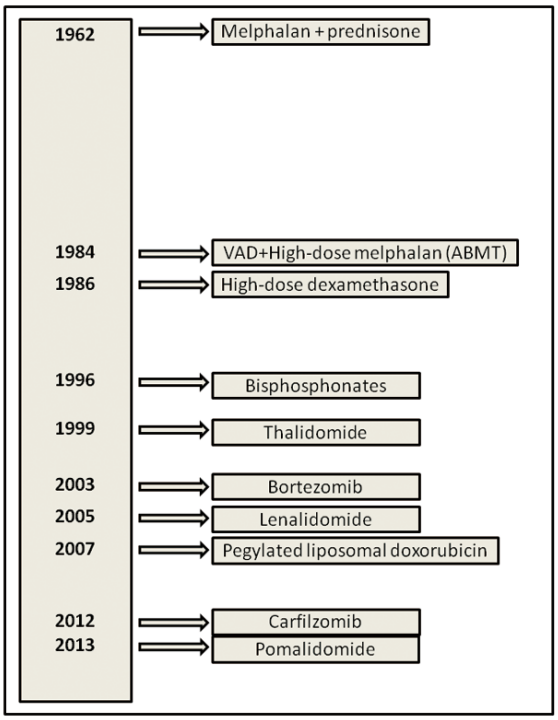

Figure 1 Progress in the treatment of multiple myeloma[127].

In patients who are not candidates for ASCT (elderly or unfit patients), the duration of initial therapy is approximately 9-18 mo for most regimens, although in the case of lenalidomide/low-dose dexamethasone (Rd), therapy is often continued until progression if the patient well tolerates the treatment[8].

ASCT INDUCTION THERAPY

Bortezomib-based regimens

The addition of novel agents into the induction regimens significantly improved the outcome of patients with newly diagnosed MM[9].

Bortezomib (Figure 2), alone or in combination with dexamethasone (VD), is active in newly diagnosed myeloma patients[10]. VD shows superior response rates when compared with vincristine/doxorubicin/prednisone (VAD), with a VGPR rate of 38% *vs* 15% after induction therapy in younger patients. The higher VGPR rate was confirmed after transplantation (54% *vs* 37%), with a PFS improvement (36 mo *vs* 30 mo)[11]. In spite of these good results, no OS benefit was noticeable.

The three-drug combination bortezomib/thalidomide/dexamethasone (VTD) has been compared with thalidomide/dexamethasone (TD) or VD[12,13]: VTD resulted in better response rates and PFS, but no OS benefit was observed. Nevertheless, these studies evidenced the ability of VTD plus double ASCT followed by bortezomib-based consolidation to overcome the poor prognostic effects of t(4;14) translocation[13]. VTD is particularly useful in patients with acute renal failure since it acts rapidly and can be used without dose modification[13].

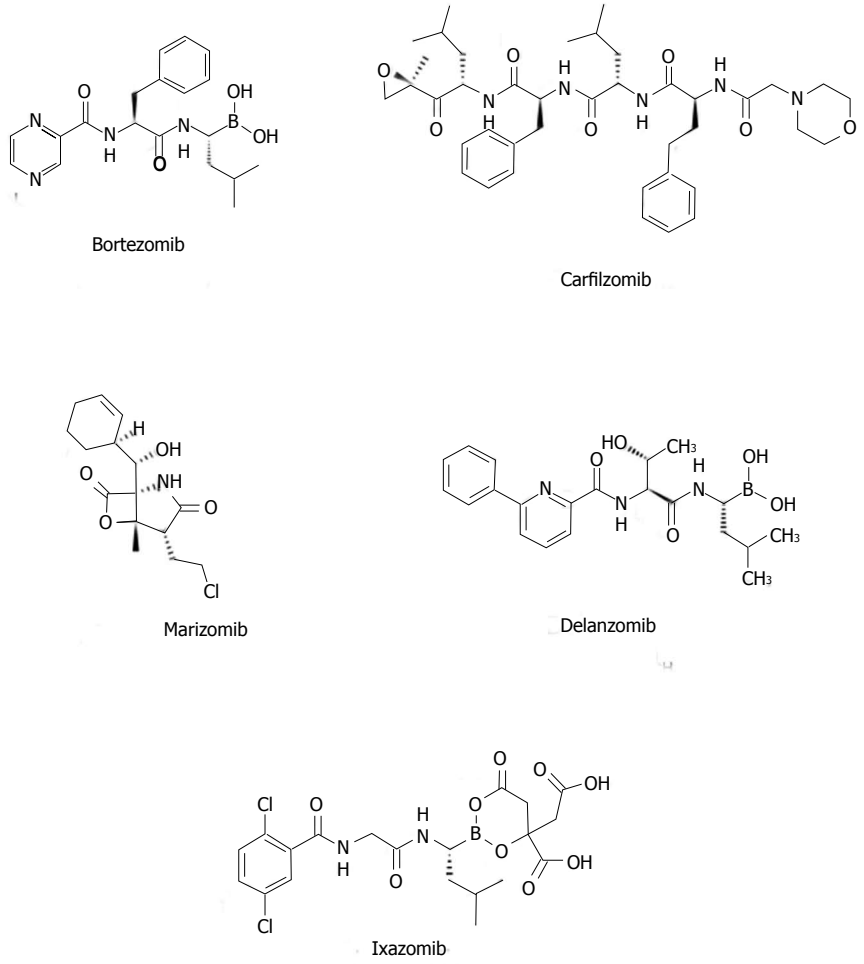

Figure 2 Chemical structure of bortezomib and new proteasome inhibitors carfilzomib, delanzomib (CEP18770), marizomib (NPI-0052) and ixazomib (MLN9708).

Other two combinations can represent excellent choices when considering a bortezomib-containing regimen as frontline therapy in newly diagnosed MM patients: the three-drug combination bortezomib/cyclophosphamide/dexamethasone (CyBorD or VCD) and the four-drug combination bortezomib/cyclophosphamide/lenalidomide/dexamethasone (VCRD)[14]. The EVOLUTION trial[15], a randomized phase 2 trial in newly diagnosed myeloma patients, showed that VCD is well tolerated with similar activity compared with the combination bortezomib/lenalidomide/dexamethasone (VRD), a combination which produces remarkably high overall and complete response rates[16]. In this trial, CR was achieved in 22% and 47% of patients treated with two different schedules of VCD *vs* 24% of patients treated with VRD. Although highly active, VCRD had similar CR rates compared with either VCD or VRD (Table 1).

Immunomodulator-based regimens

Thalidomide and its derivate lenalidomide are designed as "immunomodulatory" drugs (Figure 3). Recent studies have demonstrated that the mechanism of action of these drugs may be more complex. These drugs target both tumor plasma cells and their microenvironment[17]. Their activity seems to be mediated through cereblon, the putative primary teratogenic target for thalidomide[18].

In newly diagnosed MM, TD produces response rates of 65%-75%[19,20]. Two randomized trials found TD to be superior to dexamethasone alone[21]. Patients receiving thalidomide-based regimens require deep vein thrombosis prophylaxis with aspirin, low-molecular weight heparin or Coumadin[22].

In the transplant setting, there are some trials which aim to clarify the role of lenalidomide as induction therapy[23]. Although its use during induction determines good response rates, it seems to impact on the mobilization of stem cells[24,25]. Therefore, in patients over the age of 65 and those who have received more than 4 cycles of Rd, stem cells must be mobilized with either cyclophosphamide + Granulocyte Colony-Stimulating Factor or with plerixafor[26].

Other combinations (also with bortezomib and alkilators) have been already discussed in the previous paragraph (Table 1).

ELDERLY/UNFIT PATIENTS

Bortezomib-based regimens

In the phase 3 VISTA trial, the combination of bortezo-

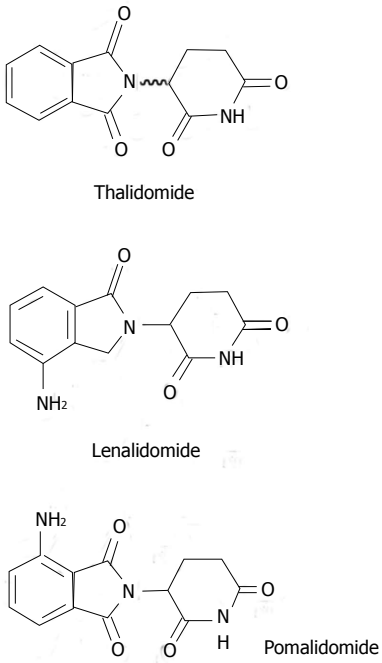

Figure 3 Chemical structures of thalidomide and its analogues, lenalidomide (CC-5013) and pomalidomide (CC-4047).

mib/melphalan/prednisone (VMP) was associated with significantly improved OS compared with melphalan/prednisone (MP). OS persisted with a long-term follow-up[27,28]. In a subsequent randomized trial, there was no significant advantage of bortezomib/thalidomide/prednisone (VTP) over VMP[29]. The principal toxicity of VMP is peripheral neuropathy, which can be reduced using a once-weekly regimen[29,30]. Moreover, subcutaneous administration of bortezomib also reduces the incidence of neurophaty and other toxicities[31].

The combination bortezomib/melphalan/prednisone/thalidomide (VMPT) has been compared with VMP in a randomized phase 3 trial conduct by the Italian group GIMEMA[30]. The 3-year PFS rate was 56% with VMPT compared to 41% with VMP. However, patients in the VMPT arm received maintenance therapy with bortezomib and thalidomide, whereas patients in the VMP arm did not receive any additional therapy beyond 9 mo. Furthermore, no OS differences were observed: the 3-year OS was 89% with VMPT and 87% with VMP. Therefore, it seems that there is no advantage using VMPT as initial therapy (Table 1).

Immunomodulator-based regimens

It has been demonstrated that TD is inferior in terms of activity and toxicity when compared to lenalidomide-based regimens. In a Mayo Clinic study of 411 newly diagnosed patients lenalidomide plus dexamethasone was significantly superior to TD in terms of response rates, PFS, and OS[32]. Moreover, in another phase 3 study, the OS with TD was inferior to MP in newly diagnosed elderly patients[33]. This combination can be considered an option for initial therapy only in patients with acute renal failure, and in combination with bortezomib.

Table 1 Bortezomib-based induction, immunomodulatory-based induction, bortezomib-based and immunomodulatory-based regimens for transplant-elegible patients

Regimen	Drugs	Ref.
Bortezomib-based induction		
VD	Bortezomib/dexamethasone	[12,13]
VTD	Bortezomib/thalidomide/dexamethasone	[12,13]
CyBorD or VCD	Bortezomib/cyclophosphamide/dexamethasone	[14-16]
VCRD	Bortezomib/cyclophosphamide/lenalidomide/dexamethasone	[14-16]
VRD	Bortezomib/lenalidomide/dexamethasone	[14-16]
Immunomodulatory-based induction		
TD	Thalidomide/dexamethasone	[19,20]
VTD	Bortezomib/thalidomide/dexamethasone	[12,13]
Rd	Lenalidomide/low-dose dexamethasone	[23]
VCRD	Bortezomib/cyclophosphamide/lenalidomide/dexamethasone	[14-16]
VRD	Bortezomib/lenalidomide/dexamethasone	[14-16]
Bortezomib-based		
VMP	Bortezomib/melphalan/prednisone	[29-31]
VMPT	Bortezomib/melphalan/prednisone/thalidomide	[30]
Immunomodulatory-based		
TD	Thalidomide/dexamethasone	[32]
MPT	Melphalan/prednisone/thalidomide	[40,41]
Rd	Lenalidomide/low-dose dexamethasone	[44]
RD	Lenalidomide/high-dose dexamethasone	[43]
MPR	Melphalan/prednisone/lenalidomide	[45]

MPT has shown better response rates compared with MP in six randomized studies[34-39]. In four of these trials a significant prolongation of PFS with MPT has been observed[34-38], and an OS advantage has been observed in three of them[34,35,38]. The evident superiority of MPT over MP has been confirmed by two meta-analyses of these randomized trials[40,41].

Grade 3/4 adverse events occur in approximately 55% of patients treated with MPT compared with 22% in patients treated with MP and 43% with TD. There is a considerable risk (20%) of deep vein thrombosis with MPT, confirming the necessity of thromboprophylaxis in these patients.

Rd is active in newly diagnosed myeloma patients[42]. In a randomized trial, Rd demonstrated less toxicity than lenalidomide plus high-dose dexamethasone (RD)[43] with better patient's compliance and OS.

As a result, high-dose dexamethasone is no longer recommended also because its toxicity makes it difficult to incorporate it into combination regimens. Rd is also an attractive option for the treatment of elderly patients with newly diagnosed myeloma because of its excellent tolerability, convenience, and efficacy. The 3-year OS rate with Rd in patients older than 70 who did not receive ASCT is 70%[44], and is comparable to results with MPT and VMP. All patients receiving Rd require antithrombosis prophylaxis with aspirin; low-molecular-weight heparin or Coumadin is mandatory in patients with high risk of DVT[22].

The combination melphalan/prednisone/lenalidomide (MPR) has been recently compared with MP in a

Table 2 Combinations of more new drugs for elderly or transplant-inelegible patients

Regimen	Drugs	Ref.
VDT-PACE	Bortezomib/dexamethasone/thalidomide/cisplatin/doxorubicin/cyclophosphamide/etoposide	[46]
VCRD	Bortezomib/cyclophosphamide/lenalidomide/dexamethasone	[15,16]
VRD	Bortezomib/lenalidomide/dexamethasone	[15,16]

randomized trial of patients 65 years of age or older with newly diagnosed MM[45]. The median PFS was similar between MPR and MP, 14 mo *vs* 13 mo, respectively. The disappointing lack of improvement in PFS with MPR compared with MP may be related to the fact that dose reductions of both melphalan and lenalidomide are often required when the 2 agents are combined (Table 1).

Combinations of new drugs

Multiagent combination regimens such as bortezomib/dexamethasone/thalidomide/cisplatin/doxorubicin/cyclophosphamide/etoposide (VDT-PACE) have been tested extensively at the Myeloma Institute for Research and Therapy at Arkansas[46]. VDT-PACE is particularly useful in patients with aggressive disease, such as plasma cell leukemia or multiple extramedullary plasmacytomas.

The EVOLUTION trial, which compared VCD with VCRD and VRD[15,16], evidenced similar activity and CR rates for all the three combinations in newly diagnosed myeloma patients (Table 2).

CHOICE OF INITIAL THERAPY

MM treatment strategies have largely changed in recent years with numerous new combinations available for initial approaches. A plethora of randomized data enable physicians to choose the best regimen for initial therapy, but most available randomized trial data are comparisons of newer regimens with older alkylator- or anthracycline-based regimens. Only few trials have compared modern regimens between each other, and in these studies surrogate end points, such as response rates and PFS, were used. No randomized data with OS or patient-reported quality-of-life end points are available. PFS cannot represent a good surrogate for clinical benefit. In numerous instances, PFS has proven to be a poor indicator of clinical benefit: the arm with PFS advantage showed[11,14] inferior OS[43]. The choice of therapy is driven by surrogate end points such as CR or PFS that were primary end-points of worldwide randomized trials. OS or patient-reported quality-of-life remain the primary end-points that should drive the rational choice of treatment when a patient with MM needs initial therapy.

Moreover, an optimal treatment strategy for newly diagnosed myeloma patients should be also based on the evaluation of risks and benefits[47]. The actual "cure *vs* control" debate on whether we should treat myeloma with an aggressive multidrug strategy targeting CR or a sequential disease control approach is concentrated on this aspect[47,48]. The risk-adapted stratification of patients can be a good indicator for personalized treatments[49].

In standard-risk patients VCD seems to be a reasonable option for initial therapy. Higher CR rates, low toxicity, and the lack of any adverse effect on stem cell mobilization are the principal advantages compared to immunomodulator-based regimens. The risk of neurotoxicity early in the disease course represents the major unfavorable factor. However, recent studies show that the neurotoxicity of bortezomib can be greatly diminished by administering bortezomib on a once-weekly schedule[39,40] or by administering the drug subcutaneously[50].

There are no data showing that more expensive regimens (*e.g.*, VRD) are safer or more effective in terms of OS or quality-of-life compared to Rd or VCD (or VTD). Although in elderly patients, melphalan-containing triplet regimens such as VMP and MPT have proven efficacy over MP, it is not clear whether they are superior to non-melphalan-containing regimens.

In intermediate or high-risk patients (particularly t(4;14) translocation), bortezomib-containing regimens followed by double ASCT and eventually mantainance therapy are able to overcome the poor prognosis with good CR rates and sustained CR[13,51]. Therefore, VCD or a similar bortezomib-containing regimen would be the preferred choice in this subset of patients.

VRD is a rather expensive regimen that, although response rates and CR rates are very high, cannot be suggested as standard therapy outside clinical trials because of the lack of data from randomized trials comparing its safety and efficacy. However, it may represent a reasonable treatment option in high-risk myeloma patients since other current options appear inadequate.

At present, no sufficient data are available to recommend any quadruple regimen as initial therapy outside of clinical trials. VDT-PACE may be a good choice in very high-risk patients with extensive extramedullary disease or plasma cell leukemia at the time of initial diagnosis[46].

In patients with acute renal failure due to suspected light-chain cast nephropathy, VCD (or VTD) is of particular value and is preferred as initial therapy[52].

In patients who are not candidates for ASCT, melphalan-containing regimens such as MPT, VMP, and MPR are indicated. Most patients with newly diagnosed MM are > 65 years old with 30% > 75 years.

Finally, elderly patients are more susceptible to side effects and may be unable to tolerate full drug doses[53]. For these patients, lower dose-intensity regimens improve the safety profile and thus optimize treatment outcomes. The occurrence of serious hematological and non-hematological adverse events during treatment should be carefully taken into account to adjust doses and optimize the outcome.

RELAPSED/REFRACTORY MM

In the relapsed/refractory setting, conventional or high-dose chemotherapy has been a longstanding approach to salvage treatment. The regimens include high-dose melphalan; high-dose methylprednisolone; high-dose dexamethasone; VAD; vincristine/melphalan/cyclophosphamide/prednisone alternating with vincristine/carmustine/doxorubicin/prednisone; doxorubicin/vincristine/dexamethasone/etoposide/cyclophosphamide; cisplatin/doxorubicin/cyclophosphamide/etoposide (DT-PACE); dexamethasone/cyclophosphamide/etoposide/cisplatin (DCEP)[54-61]. Overall response rates for salvage combination chemotherapy are between 25% and 65%, with morbidity and mortality related to the intensity of therapy.

Allogeneic transplant shows limited clinical benefit for the treatment of relapsed/refractory MM with long-term, disease-free survival of 10% to 20% and high toxicity. Few patients, even those with poor-risk disease, are ultimately cured with this approach[62,63].

Second autologous transplantation may be beneficial and safe for some patients with relapsed/refractory disease. Moreover, the overall response rates in recent studies with small sample sizes range from 55% to 69%, with a 100-d mortality rate of 10%[64,65].

Thalidomide was the first novel agent to be evaluated in patients with relapsed/refractory MM. Several studies have demonstrated the effectiveness of thalidomide as a single agent producing partial response or better (> PR) in 30% of patients with relapsed/refractory disease, with a 1-year OS rate of 60% and median OS of 14 mo[66]. In the phase Ⅲ OPTIMUM study, different doses of thalidomide (100 mg/d, 200 mg/d, or 400 mg/d) demonstrated no difference in response rate and OS[67]. Thalidomide has been successfully combined with multiple conventional cytotoxic agents for the treatment of relapsed/refractory MM. When compared with thalidomide alone, the addition of dexamethasone resulted in higher response rates of about 50%[68]. The addition of cyclophosphamide to thalidomide with or without dexamethasone led to higher responses (> PR: 57%-84%)[69,70]. Evidence also suggests that the efficacy of thalidomide in relapsed/refractory MM may be improved when combined with melphalan (> PR: 59%), MP (> PR: 42%), melphalan/dexamethasone (> PR: 70%), pegylated liposomal doxorubicin/dexamethasone (> PR in 76%), pegylated liposomal doxorubicin/vincristine/dexamethasone (> PR: 75%), DT-PACE (> PR: 32%), or cyclophosphamide/etoposide/dexamethasone (TCED; > PR: 68%)[71-75].

Monotherapy with bortezomib demonstrated response rates of 25% to 35% in patients with relapsed/refractory MM in initial phase I and Ⅱ studies[76,77]. The randomized phase Ⅲ APEX study demonstrated a survival benefit with bortezomib compared to high-dose dexamethasone in patients who had received a median of two prior therapies[78]. Bortezomib-based treatment demonstrated superior response rates (43% *vs* 18%; $P < 0.001$), TTP (6.2 mo *vs* 3.5 mo; $P < 0.001$), and 1-year OS (80% *vs* 66%; $P < 0.003$) compared with dexamethasone[78]. The benefit of 6-mo OS for patients who received bortezomib persists despite substantial crossover (> 62%) from dexamethasone to bortezomib[78]. The addition of dexamethasone to bortezomib resulted in response improvement in 18% to 34% of patients[78]. Multiple chemotherapeutic agents have been successfully combined with bortezomib in relapsed/refractory MM, such as the combination with pegylated liposomal doxorubicin (> PR: 44%), low-dose dexamethasone/pegylated liposomal doxorubicin (PAD) (> PR: 67%-85%), oral or intravenous melphalan (> PR: 47%-68%), and low-dose cyclophosphamide/prednisone/dexamethasone (> PR: 68%- 82%)[79-82].

Lenalidomide as a single agent at the maximum dose of 25 mg once daily determined response rates ranging from 29% to 39% in patients who had received a median of three prior therapies[83].

MM-009 and MM-010 trials comparing RD *vs* dexamethasone alone demonstrated improved overall response (60.6% *vs* 21.9%; $P > 0.001$), TTP (13.4 mo *vs* 4.6 mo; $P > 0.001$), and OS (38.0 mo *vs* 31.6 mo; $P > 0.045$) for patients treated with RD[84-86]. Crossover to RD for patients who previously received dexamethasone alone (41.9%) did not modify the results. Moreover, the RD combination also appears to be effective in very elderly (> 75 years) patients with relapsed MM, demonstrating overall response rates of 62% and median PFS of 14 mo[87]. Currently, all new dexamethasone combination trials use low-dose dexamethasone instead of high-dose dexamethasone.

Lenalidomide has also demonstrated efficacy in combination with doxorubicin/dexamethasone (RAD; > PR: 73%), low-dose cyclophosphamide/prednisone (REP; minimal response or better > MR: 64.3%), cyclophosphamide/dexamethasone (> MR: 75%), and pegylated liposomal doxorubicin/vincristine/dexamethasone (> PR: 75%) in patients with relapsed/refractory MM[88-91].

Numerous studies have evaluated the combination of two established novel agents with conventional and/or cytotoxic drugs in the relapsed/refractory setting[92-97]. Bortezomib has been successely combined with thalidomide and other drugs: VTD; >PR: 63%, PAD > PR: 74%, VCTD; > PR: 88%, VMPT; > PR: 67%, and VMDT; > PR: 66%. Other studies have evaluated the combination of VRD with good response rates (> MR: 61%-86%) even in patients resistant to thalidomide, lenalidomide, or bortezomib[1] (Table 3).

CHOICE OF THERAPY AT RELAPSE

Decisions regarding treatment at relapse should be made considering the timing of relapse, the efficacy and toxicity of drugs used in prior therapies, age, bone marrow and renal function, co-morbidities and patient's preference. Also, it should be recognized that it is not necessarily best practice to refer particular therapies at specified time points. In the future, it is likely that therapy will be 'risk-adapted' and the presence or absence of specific prog-

Table 3 Combinations for relapsed/refractory patients

Regimen	Drugs	Ref.
MEL	High-dose melphalan	[54,55]
PDN	High-dose methylprednisolone	[56,57]
DEX	High-dose dexamethasone	[57]
VAD	Vincristine/ doxorubicin/prednisone	[58]
VMPC	Vincristine/melphalan/cyclophosphamide/ prednisone	[58]
VBAP	Vincristine/carmustine/doxorubicin/ prednisone	[58]
CEVAD	Doxorubicin/vincristine/dexamethasone/ etoposide/cyclophosphamide	[59]
DCEP	Dexamethasone/cyclophosphamide/ etoposide/cisplatin	[60]
DT-PACE	Cisplatin/doxorubicin/cyclophosphamide/ etoposide	[61]
AlloSCT	Allogeneic transplant	[62,63]
ASCT	Second autologous transplant	[64,65]
THAL	Thalidomide monotherapy	[66,67]
TD	Thalidomide/dexamethasone	[68]
TC	Thalidomide/cyclophosphamide	[69]
TCD	Thalidomide/cyclophosphamide/ dexamethasone	[70]
MPT	Melphalan/prednisone/thalidomide	[71]
MTD	Melphalan/thalidomide/dexamethasone	[72]
TAD	Thalidomide/pegylated liposomal doxorubicinxorubicin/dexamethasone	[73]
TAVD	Thalidomide/pegylated liposomal doxorubicinoxorubicin/vincristine/ dexamethasone	[74]
TCED	Thalidomide/cyclophosphamide/etoposide/ dexamethasone	[75]
BOR	Bortezomib monotherapy	[76-78]
VD	Bortezomib/dexametasone	[78]
VA	Bortezomib/pegylated liposomal doxorubicin	[79]
PAD	Bortezomib/low-dose dexamethasone/ pegylated liposomal doxorubicin	[80]
VM	Bortezomib/melphalan	[81]
VCD	Bortezomib/low-dose cyclophosphamide/ prednisone/	[82]
LEN	Lenalidomide monotherapy	[83]
RD	Lenalidomide/dexamethasone	[84-87]
RAD	Lenalidomide/doxorubicin/dexamethasone	[88,89]
REP	Lenalidomide/low-dose cyclophosphamide/ prednisone	[90]
RCD	Lenalidomide/cyclophosphamide/ dexamethasone	[91]
RAVD	Lenalidomide/pegylated liposomal doxorubicin/vincristine/dexamethasone	[1]
PATD	Bortezomib/ pegylated liposomal doxorubicin/thalidomide/dexamethasone	[92]
VMDT	Bortezomib/melphalan/dexamethasone/ thalidomide	[93]
VMPT	Bortezomib/melphalan/prednisone/ thalidomide	[94]
VTD	Bortezomib/thalidomide/dexamethasone	[95]
PAD	Bortezomib/pegylated liposomal doxorubicin/dexamethasone	[1]
VCTD	Bortezomib/cyclophosphamide/ thalidomide/dexamethasone	[1]
VCRD	Bortezomib/cyclophosphamide/ lenalidomide/dexamethasone	[1]
VRD	Bortezomib/lenalidomide/dexamethasone	[1]

nostic factors may determine the choice of therapy both at diagnosis and relapse. Patients with a long first plateau phase after their initial therapy may be treated with the same first-line regimen because they are likely to respond again at relapse. The use of a second ASCT is a possible choice according to patient's fitness and if the candidate patient had a good response to the initial transplant procedure (> 18-mo PFS).

For patients presenting with renal failure, strong consideration of treatments with a bortezomib-containing regimen should be given: it determines a rapid reduction in light chain load to the kidneys, and maximizes chances of regaining renal function.

Entry into clinical trials should be considered at each relapse.

MAINTENANCE THERAPY FOR MM

Maintenance therapy is actually one of the most controversial debates in the care of patients with MM. Historically, this has been attempted with corticosteroids, interferon, and thalidomide. PFS, but not OS, was often prolonged and was frequently accompanied by significant morbidity and high discontinuation rates[98,99].

Lenalidomide is a more tolerable immunomodulatory drugs (IMiD). Maintenance therapy with this drug has been explored in recent randomized, placebo-controlled trials. Two studies[100,101] evaluated the role of maintenance lenalidomide therapy in patients after ASCT compared with placebo, and a third evaluated extended-use lenalidomide in older patients initially treated with melphalan-based regimens[45]. Only 1 out of the 3 has shown a survival benefit.

In a recent trial comparing PAD with VAD, patients randomized to the PAD arm received maintenance with bortezomib (every 2 wk) after ASCT, and those in the VAD arm received thalidomide as maintenance[102].

Currently available data are not sufficient to recommend routine maintenance with lenalidomide for all patients outside clinical trials, but it can be considered in subgroups of patients in whom the benefits appear to outweigh the risks (*e.g.*, standard-risk patients who are known to be lenalidomide responsive and are not in VGPR or better after completion of initial therapy).

In intermediate- and high-risk myeloma patients, a bortezomib-based maintenance approach may be preferable but needs further studies. Preliminary results are encouraging and suggest improved PFS and OS with bortezomib maintenance, but it is not clear if this can be attributed to differences in induction or maintenance therapy.

NEWEST DRUGS

Pomalidomide

The IMiD pomalidomide (Figure 3) exerts its anticancer effects throught the angiogenesis inhibition, immunomodulation, impeding cytokine production, and interaction with the bone marrow and tumor microenvironment dependent on Cereblon expression[103]. Decreased cereblon mRNA expression has been correlated with lenalidomide resistance[18,103]. Interestingly, pomalidomide

appears to remain effective in lenalidomide resistant cells[103]. Pomalidomide is the most potent IMiD, having 100 times strength of thalidomide and 10 times that of lenalidomide[104]. Moreover, the proliferation and survival of myeloma cells are largely unaffected by thalidomide, whereas lenalidomide and pomalidomide cause both cell cycle arrest and apoptosis[105]. Specifically, they induce cell cycle arrest by P21 WAF activation independently of P53. This highlights the possibility of using these agents to treat P53 mutated malignancies[106].

Pomalidomide has shown good results in phase I studies with 50% of patients achieving at least a PR and 10% achieving a CR at a maximum tolerated dose of 4 mg for 21 or 28 d [107,108]. In the MM-002 (a randomized phase Ⅰ/Ⅱ open-label dose escalation study) heavily treated patients refractory to both lenalidomide and bortezomib were included. Efficacy results in 21% of patients achieving at least a PR and in patients refractory to both lenalidomide and dexamethasone, 25% had a response[109].

Phase Ⅱ studies demonstrated improvement of the response rate and quality of responses when dexamethasone was associated to pomalidomide in heavily pretreated patients[110]. Responses were observed in > 60% of patients (5% CR, 30% VGPR, and 30% MR) and in 40% of lenalidomide-refractory patients, 40% of thalidomide-refractory patients, and 60% of bortezomib-refractory patients. In high-risk patients (plasma cell labeling index ⩾ 3%, deletion 17p, t(4;14) or t(14;16) by fluorescent *in situ* hybridization, or deletion 13 on conventional cytogenetics) responses were observed in 74% of cases. Also in extramedullary disease in the phase Ⅱ study conducted at the Mayo Clinic[111] the response rate was 31%, with two patients achieving CR and two patients achieving PR.

Two sequential phase Ⅱ studies of patients refractory to both bortezomib and lenalidomide conducted at the Mayo Clinic[112], in which two dosing strategies were compared, suggest that 4 mg daily dosing does not yield superior responses than 2 mg daily dosing: the 2 mg cohort had an MR or better (49%) while the 4 mg group had an overall response rate of 43; 26% of patients achieved at least a PR in both cohorts. In contrast, a trend towards a dose-dependent response was observed in the phase I MM-002 study[113]. Toxicities are similar at the 2 and 4 mg dose levels. Neutropenia is the major toxicity described (26%-66% grade 3/4), thrombocytopenia and anemia are also common (13% and 17% grade 3/4, respectively)[112].

Nonhematologic toxicities are seen in 5% of patients: fatigue in 62% of cases (8% grade 3/4), thromboembolic events in 3%, and peripheral neuropathy in 13% (up to 33% in patients with pre-existing neuropathy that worsens). Acute noninfectious pulmonary toxicity has been described in two patients[113].

Carfilzomib

Carfilzomib (also known as PR-171; Figure 2) is a new stable and irreversible proteasome inhibitor that is potentially more efficacious and less toxic. It inhibits the chymotrypsin-like site of the proteasome, and, at high doses, it shows additional inhibitory effects on the trypsin-like and caspase-like sites[114].

Phase I trials demonstrated sustained proteasome inhibition with two different schedules: escalating doses on 5 consecutive days, followed by a 9-d rest period in a 14-d cycle[115], and on days 1, 2, 8, 9, 15 in a 4-wk cycle[116]. The first schedule evidenced a minimal effective dose at 11 mg/m^2 and a maximal tolerated dose at 15 mg/m^2. In the second schedule a maximal tolerated dose was not reached and the highest dose administered was 27 mg/m^2. The most common side effects were thrombocytopenia and febrile neutropenia (carfilzomib-associated dose-limiting toxicities), low-grade fatigue and nausea, but no significant peripheral neuropathy was observed and no aggravation in patients with preexisting peripheral neuropathy was reported.

In bortezomib-naive patients, carfilzomib showed an impressive single-agent ORR of 52%[117]. Carfilzomib has shown single-agent activity in heavily pretreated MM patients (80% were double refractory) in the twice-weekly regimen with dose-reduction (20 mg/m^2) in the first cycle to abrogate potential tumor-lysis syndrome[118]. The dose was escalated to 27 mg/m^2 in cycle 2. The response rate was 23.7%, with a median duration of response of 7.8 mo, a PFS of 3.7 mo, and an OS of 15.6 mo. Clinical benefit was observed in one-third of the patients. Outcome was not influenced by adverse cytogenetics, renal impairment, disease stage, or Eastern Cooperative Oncology Group performance score. Drug-related adverse events (AEs) of all grades were most frequently fatigue (37%), nausea (34%), and thrombocythemia. Grade 3/4 hematologic AEs included anemia (24%), thrombocytopenia (29%), lymphopenia (20%), and neutropenia (11%). Grade 3/4 nonhematologic AEs included pneumonia (9%), hyponatremia (8.3%), fatigue (7.5%), and hypophosphatemia (6.0%). Treatment-emergent peripheral neuropathy was uncommon (12.4%) and considered to be carfilzomib related in only 8.3% of patients. Dose adjustment for renal insufficiency was not necessary[119].

The extension of the infusion time to 30 min allowed tolerance of higher doses of carfilzomib with good ORR[120].

Improvement of the response has been obtained with the association of lenalidomide and low-dose dexamethasone to carfilzomib[121,122]. The overall response was 78%-98% with an impressive stringent complete remission rate of 42% after a median of 12 cycles of therapy.

In newly diagnosed elderly patients the combination with melphalan and prednisone (CMP) administered on the usual schedule twice weekly with a 12-d rest in a 42-d cycle was safe and effective with a response rate of 92% at the interim analysis[122].

The cyclophosphamide/carfilzomib/thalidomide/dexamethasone demonstrated a response rate of 100% in newly diagnosed MM patients[123].

FUTURE DIRECTIONS

Our understanding of the transformation of normal

plasma cells into malignant myeloma cells is improving[124]. The interaction between MM cells and their microenvironment is the focus of intense research[125] and several targets have emerged from these studies[126,127]. In addition, drugs that target the cell cycle, membrane receptors, immunomodulators and antiangiogenics are also being investigated in MM.

New proteasome inhibitors

Delanzomib (CEP18770), Marizomib (NPI-0052) and Ixazomib (MLN9708), (Figure 2) are currently being evaluated in phase I and II studies[128]. Marizomib (NPI-0052) is an orally active proteasome inhibitor and a more potent inhibitor of the NF-kB and other cytokines than bortezomib[128]. It is able to overcome bortezomib resistance both *in vitro* and *in vivo*. It interferes with the chymotryptic-like, tryptic-like and caspase-like proteolytic activity of the proteasome. Studies are currently evaluating Marizomib as a single agent as well as in combination with bortezomib since a synergistic effect has been observed[129].

Histone deacetylase inhibitors

The expression of genes is controlled by the DNA/histone interaction. Excessive deacetylated level of histones has been linked to cancer pathologies by promoting the repression of tumor suppressor genes. Overexpression of histone deacetylase (HDAC) by MM cells results in decreased DNA transcription, including transcription of tumor suppressor genes, and inhibition of HDAC reverses these effects[130]. HDAC inhibitors mediate tumor cell death via caspase-dependent and independent apoptosis and autophagy[131], induce cell cycle arrest via p21 up-regulation, block the aggresome complex and induce cell death via the accumulation of ubiquitinated proteins[132].

Currently several HDAC inhibitors have being studied as single agents or in combination with other agents mainly bortezomib and lenalidomide. Vorinostat (SAHA) is an oral HDAC inhibitor that down-regulates IGF-1 and IL-6 signaling pathways as well as DNA synthesis and repair enzymes[133].

In phase I trials in patients with relapsed/refractory MM disease oral vorinostat was well tolerated. Side effects included fatigue, anorexia, diarrhea and nausea. The combination with bortezomib has shown promising activity[134]. On 23 patients with a median of 7 prior regimens the response rate was 42%, including 3 cases of PR among 9 bortezomib-refractory patients.

A second HDAC inhibitor tested in combination with other therapies for relapsed/refractory MM is Panobinostat (LBH589), a potent pan deacetylase inhibitor that disrupts aggresome and HSP90 function *via* inhibition of HDAC6 and promotes cytotoxic misfolded/unfolded protein aggregates and MM cell death[132]. In a phase I study response rate was 68% (26/38 pts) in patients across all cohorts and, particularly, 62% (8/13 pts) in bortezomib-refractory patients. Major adverse events included grade 3/4 thrombocytopenia (n = 30), neutropenia (n = 23), diarrhea (n = 23), nausea (n = 18), pyrexia (n = 17) and fatigue (n = 16). No grade 3/4 peripheral neuropathy was observed. Various phase Ⅰ, Ⅱ and Ⅲ studies are ongoing to establish the role of panobinostat and its combinations (*i.e.*, plus bortezomib/dexametasone, lenalidomide/dexamethasone, melphalan/prednisone/thalidomide, everolimus, carfilzomib) in MM treatments.

Other HDAC inhibitors are currently being evaluated in the treatment of MM. Romidepsin, Belinostat, ITF2357 and AR 42 are still under investigation.

AKT inhibitors

The PI3K/AKT pathway is a central signaling pathway in several cellular functions including proliferation, growth, survival and migration. AKT activation induces growth and survival advantage to MM cells through GSK-3β and mTOR phosphorylation and has been shown to be associated with advanced stage and poor prognosis in MM patients and resistance to dexamethasone in MM cells[135].

Perifosine (KRX-0401) is a novel synthetic oral AKT inhibitor[136]. On the basis of a phase Ⅰ/Ⅱ study the combination of perifosine and bortezomib with or without dexamethasone showed a 41% response rate (65% in the bortezomib-group *vs* 32% in the bortezomib-refractory group)[137] with manageable toxicity. A phase Ⅲ randomized trial evaluating the same combinations in patients with relapsed/refractory MM previously exposed to bortezomib is ongoing.

Mammalian target of rapamycin inhibitors

The Mammalian target of rapamycin (mTOR) is an intracellular serine-threonine kinase that controls cell growth, proliferation, motility, survival and metabolism. mTOR exerts its downstream effects through the formation of protein complexes called mTORC1 and mTORC2 with different functions and target molecules[138]. mTOR acts as a neoplastic switch that is frequently turned on by many mutations found in cancer and, hence, inhibition of mTOR and its complex offers a promising target.

In a phase Ⅱ study, temsirolimus showed a 38% response rate in 16 patients with relapsed/refractory MM with a TTP of 138 d and a good toxicity profile[139]. The combination of temsirolimus with bortezomib obtains a PR or better in 33% of patients[140]. Everolimus (RAD001) has also been studied in MM as a single agent and in combination with lenalidomide, with no encouraging preliminary results. Other mTOR inhibitors such as ridaforolimus are currently being evaluated for their anti-MM activity in preclinical phase Ⅰ studies.

Emerging data have shown that rapamycin analogs as well as mTOR inhibitors that have activity against mTORC2 do not appear to be effective as monotherapy (only SD or MR obtained in clinical trials with rare PR). They may represent agents that could be evaluated in combination with other anti-myeloma agents in the future.

Heat shock protein 90 inhibitors

Heat shock protein 90 (HSP90) is a molecular chaperone that facilitates the folding and stability of numerous signaling molecules that control the growth and survival

of cancer cells[141]. The client proteins for HSP90 include transcription factors, oncogenic kinases and receptors that are associated functionally with cell cycle control and signaling, and it has been shown to be a key molecular chaperone for signal transduction proteins critical to MM cell growth, survival and drug resistance.

Preclinical studies with tanespimycin (KOS-953) (a small molecule inhibitor of HSP90) have shown a rationale for combining HSP90 inhibitors with bortezomib because of the HSP90 induction by bortezomib along with up-regulation of stress response gene transcripts. HSP90 inhibition increases bortezomib-induced apoptosis in MM cells by blocking the HSP90 stress response. Moreover, tanespimycin may also be protective against peripheral neuropathy associated with bortezomib. The combination tanespimycin/bortezomib in relapsed/refractory MM patients obtained a response rate of 41% in bortezomib-naïve, 20% in bortezomib-pretreated and 14% in bortezomib-refractory patients. Adverse events included diarrhea (60%), fatigue (49%), nausea (49%), thrombocytopenia (40%), and aspartate transaminase elevation (29%). Grade 1-2 peripheral neuropathy was seen in 21% patients, but no grade 3/4 peripheral neuropathy was observed, which is consistent with tanespimycin's neuroprotective effect[142]. Other HSP90 inhibitors are currently in early phase trials in MM.

Monoclonal antibodies

Monoclonal antibodies (MoAb) represent a new anticancer strategy[143] for patients refractory to new drugs. These patients were found to have a median OS of 9 mo[144]. Ideally, targets for therapeutic MoAbs should be specifically expressed on cancer cells but not on normal cells as demonstrated in other hematologic diseases[145].

A large variety of antigen targets have been studied in MM, which can be expressed either on myeloma cells or on components of the bone marrow microenvironment (bone marrow stromal cells and signaling molecules), but the majority of them are in preclinical phase/early clinical trials[143,146].

When employed as monotherapy, MoAbs have generally not produced impressive levels of response in patients with MM. However, preclinical and preliminary clinical results in patients with relapsed/refractory MM suggest that MAbs are likely to act synergistically with dexamethasone, immunomodulators, and bortezomib, showing the ability to overcome resistance to these drugs[147]. Moreover, substantial efforts are underway to develop antibodies conjugated to cytotoxic agents, such as calicheamicin, doxorubicin, taxanes, maytansinoids, dolastatins, and CC-1065 analogs[148].

The near future will see a novel interest in developing novel targets for antibody-based therapies for MM. BM angiogenesis has an important role in the initiation and progression of MM. Berardi *et al*[127] looked at novel mechanisms of vessel formation in patients with MM through a comparative proteomic analysis between BM endothelial cells (ECs) of patients with active MM (MMECs) and ECs of patients with monoclonal gammopathy of undetermined significance (MGECs) and of subjects with benign anemia (normal ECs). Four proteins were found to be overexpressed in MMECs: filamin A, vimentin, a-crystallin B, and 14-3-3f/d protein. Berardi *et al*[127] investigated the differences in MMEC *vs* MGEC proteome to identify new targets for MM anti-angiogenic management. They found that FLNA (filamin A), VIM (vimentin), CRYAB (a-crystallin B), and YWHAZ (14-3-3f/d protein) are constantly overexpressed in MMECs and enhanced by vascular endothelial growth factor, fibroblast growth factor 2, hepatocyte growth factor, and MM plasma cell CM. These proteins are critically involved in MMEC overangiogenic phenotype, and indeed, their silencing is anti-angiogenic.

To date we cannot establish when and in which patients the newest drugs are useful. Their use should be limited to patients enrolled in clinical trials until their conclusion and until the role of these newest drugs in myeloma therapy is clarified. Finally, the regulatory agencies (FDA for United States and EMA for Europe) should approve the regular use of these drugs before their clinical use outside of clinical trials (Table 4).

CONCLUSION

The therapy and treatment strategies for MM have largely changed in the past decade. The goal is to improve patient's survival. The evaluation of treatments must include patient's stratification and personalized therapies. At the same time, it is becoming more important to control the disease in a long-term fashion, maintaining the quality of life of the patient since it is still difficult to cure this disease.

Our understanding of MM pathogenesis including the intracellular mechanisms as well as the interactions between MM cells and the microenvironment has helped the discovery of several targets that have become the focus of drug development. Studies on the biology of MM have highlighted the need for agents which not only target the tumor cells themselves but also disrupt their supportive microenvironment in the bone marrow.

Future studies will focus on the use of these targeted agents in multidrug-combinations: this will maximize their synergism while minimizing toxicities. Several agents and combinations are currently in different phases of clinical studies and likely they will change the natural history of MM in the near future.

The ability to modify the biology of MM using such new therapies raises the question whether a change in the treatment paradigm towards continuous therapy, providing both tumor reduction and tumor suppression, is warranted.

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Table 4 Monoclonal antibodies evaluated in multiple myeloma therapy[147]

Monoclonal antibodies targeting tumor cells specific antigens	
Antigen	**MoAb**
CD20	Rituximab, Tositumomab, 20-C2-2b Veltuzumab
CS1	Elotuzumab
CD138	B-B4, BC/B-B4, DL-101, 1D4, 1.BB.210, MI15, 2Q1484, 5F7, 104-9, 281-2, nBT062-SMCL-DM1, BT062-SPDM4, nBT062-SPP-DM1
CD38	Doratumumab, MOR202
CD40	Lucatuzumab, Lorvotuzumab
IGF-1	AVE1642, AMG479 IMCA12, R15507, Figitumumab, Dalotuzumab
CD317	AHM, Defucosylated AHM, XmAb 5592
CD48	Anti-CD48 MoAb
b2 m	IgG anti-b2m, IgM anti-b2m
CD70	SGN-70
CD74	Milatuzumab
HLADR	ID09C3, 2D7-DB
CD229	Anti-CD229
GM2 ganglioside	BIW-8962
ICAM-1	BI-505
Ku	5E2
Monoclonal antibodies targeting components of bone marrow microenvironment and plasma cellr–bone marrow stromalcell interaction	
IL-6	Siltuximab, Tocilizumab, NRI, Elsilimomab, Azintrel, SANT-7
VEGF	Bevacizumab
EGFR	Cetuximab
FGFR-3	MFGR1877A
RANKL	Denosumab
Dickkopf	Anti-DKK1, BrlQ880
Activin	RAP-011, ACE-011
BAFF	Atacicept, SG1
Other potential targets	
TRAIL-R1	Mapatuzumab
TRAIL-R2	Lexatuzumab
PD-L1	CD-011
VLA-4	Natalizumab
Kininogen	C11C1

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REVIEW

New prospects in the diagnosis and treatment of immune-mediated inner ear disease

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Abstract

Autoimmune inner ear disease (AIED) represents a very fertile research field and the advancements in the understanding of this disease have a direct application not only in patients affected with this condition but also in other inner ear disorders that share the same injury mechanism, damage to the inner ear hair cells. AIED also presents many challenges that have still to be overcome. Firstly, access to the inner ear is limited, as many interventions such as biopsies can result in great irreversible damage. Secondly, there are no completely specific markers for AIED. Lack of a definitive diagnosis can result in the treatment of patients not affected with the disease and, therefore, no response. Finally, some patients become refractory to glucocorticoids and new therapies are needed. This review offers an overview of the animal models that have contributed to the understanding of AIED pathophysiology, the value of currently available diagnostic tests, and therapeutic options, with a special focus on new therapies for non responders or patients refractory to glucocorticoids. Among these new options for therapy, biological agents have been tested recently, whereas gene and stem cell therapy may have a role in the future. The intratympanic route of administration avoids the systemic side effects associated with currently used drugs, and may become a more frequent approach in the future.

Key words: Autoimmune inner ear disease; Diagnostic tests; Biologic therapy agents; Gene therapy; Stem cells

Core tip: Readers interested in inner ear pathology will find in this review a brief summary of autoimmune inner ear disease, with special focus on the major advances achieved in the knowledge of its etiology and pathophysiology, and the diagnostic and therapeutic challenges that remain and may guide research in the next few years and beyond.

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INTRODUCTION

In 1979, Brian McCabe proposed a new clinical entity which he called autoimmune sensorineural hearing loss on the basis of a clinical and diagnostic study of a series of 18 patients and the experience acquired in their treatment[1]. He defined the disease as a bilateral, generally asymmetric hearing loss that progresses in the course of weeks or months and responds to immunosuppressive therapy. This last characteristic is essential because this disease is one of the few forms of sensorineural hearing loss potentially reversible with medical treatment.

The concept of autoimmunity was not fully accepted

by the scientific community until the late fifties and early sixties. Autoimmunity is defined as an immune reaction against the body's own antigens. Although the etiology of autoimmune inner ear disease (AIED) is not well known, several etiopathogenic mechanisms that could explain this autoimmune reaction are similar to those giving rise to other autoimmune diseases. These mechanisms involve autoreactive $CD4^+$ T cells. However, there is another group of diseases, called autoinflammatory diseases, in which the chronic inflammatory reaction is not mediated by T cells. This group includes Muckle-Wells syndrome, which presents clinical features similar to those of other autoimmune diseases, and may cause sensorineural hearing loss, but this does not respond to corticosteroids and a genetic origin has been proposed[2]. The involvement of $CD4^+$ T cells is because not all autoreactive T cells are eliminated in the thymus. While this elimination process is efficient with most antigens expressed in the human body, this is not the case with the less frequent antigens, such as those expressed in the inner ear. Fortunately there are other regulatory mechanisms that prevent the activation of these T lymphocytes.

The autoimmune reaction in AIED could be initiated by an autoimmune attack when the immune system tries to protect the inner ear against infection or external insult[3]. Viruses or pathogenic bacteria can reach the inner ear from the bloodstream, cerebrospinal fluid or middle ear and could contribute to the autoimmune response by altering host molecules so that they become self-antigens[4]. Lesions caused by surgery, trauma or drugs can expose inner ear antigens to the immune system, thereby inducing an immune response against both ears. Moreover, these insults can result in permanent tissue damage which can trigger an immune response in the future.

According to the revised Witebsky postulates proposed by Rose *et al*[5], there are three levels of evidence of an autoimmune disease: direct, indirect and circumstantial. Direct evidence requires the transmission of the characteristic lesions from human to human or from human to animal. This could occur, for instance, if the clinical features are reproduced in newborns from mothers with AIED, or in animals after injecting them with antibodies detected in AIED patients. However, in AIED most of the evidence is indirect or circumstantial. Indirect evidence is based on the re-creation of the human disease in an animal model by transferring antibodies or autoimmune T cells, or on the use of animal models of multisystem autoimmune disease. Circumstantial evidence found in AIED includes a family history of autoimmune disease, coexistence with other autoimmune diseases (such as systemic lupus erythematosus, Behçet's disease, Wegener granulomatosis, relapsing polychondritis among others), predominance of certain major histocompatibility complex alleles ($DR4^-$, $cw7^+$, $cw4^+$, B35), raised immunoglobulin G antibodies titers, and clinical response to immunosuppressive therapy. In AIED patients it is impossible to obtain other circumstantial evidence such as the presence of mononuclear cell infiltrate or of antigen-antibody complex deposits in the inner ear because the inner ear cannot be biopsied, although this evidence can be detected in animal models.

The main animal models that have contributed to the understanding of the pathophysiology of AIED are as follows.

Model of experimental labyrinthitis by means of homologous or heterologous sera against cochlear tissues

This was the first animal model ever employed to study inner ear autoimmunity[6]. The results obtained have been variable, ranging from non-histopathological changes to the development of endolymphatic hydrops, edema, hemorrhage, or perivascular inflammatory infiltrates. This model employs an inner ear homogenate and cannot, therefore, characterize specific autoantigens involved in the development of AIED[7].

Model of experimental labyrinthitis by transferring activated T lymphocytes

The activation of T lymphocytes is achieved by employing both inner ear homogenates and specific peptides such as cochlin, which is highly expressed in the inner ear, or beta-tectorin[8-10].

Model of experimental labyrinthitis by autoantibodies

Monoclonal autoantibodies against inner ear cells have been generated by immunizing mice with guinea pig or chicken inner ear extracts. KHRI-3 binds to the supporting cells in the organ of Corti causing hearing loss in guinea pigs[11]. The lack of infiltrates suggests that the lesions of the stria vascularis, spiral ligament or supporting cells are mediated by antibodies or immune complexes[12].

Model of experimental labyrinthitis by keyhole limpet hemocyanin

This model was developed by Harris and coworkers and has contributed greatly to the knowledge of AIED pathophysiology. The endolymphatic sac contains immune system cells capable of inducing or reinforcing an immune response[13]. The spiral vein of modiolus is the entryway for immunologic elements (T cells, B cells, natural killer (NK) cells, polymorphonuclear cells, macrophages) that can induce a labyrinthitis that results in functional impairment with loss of sensorial cells and ultimately leads to cochlear fibrosis and osteoneogenesis (Figure 1)[14,15].

EPIDEMIOLOGY

The incidence of AIED is not well-known because there is no definitive diagnostic test. Nevertheless, it is considered to be less frequent than sudden deafness, with 1 case out of 5000-10000 people per year. Like other autoimmune diseases, it appears to be more frequent in women. It generally presents between the ages of 20 to 50 years, and it is uncommon in childhood[16].

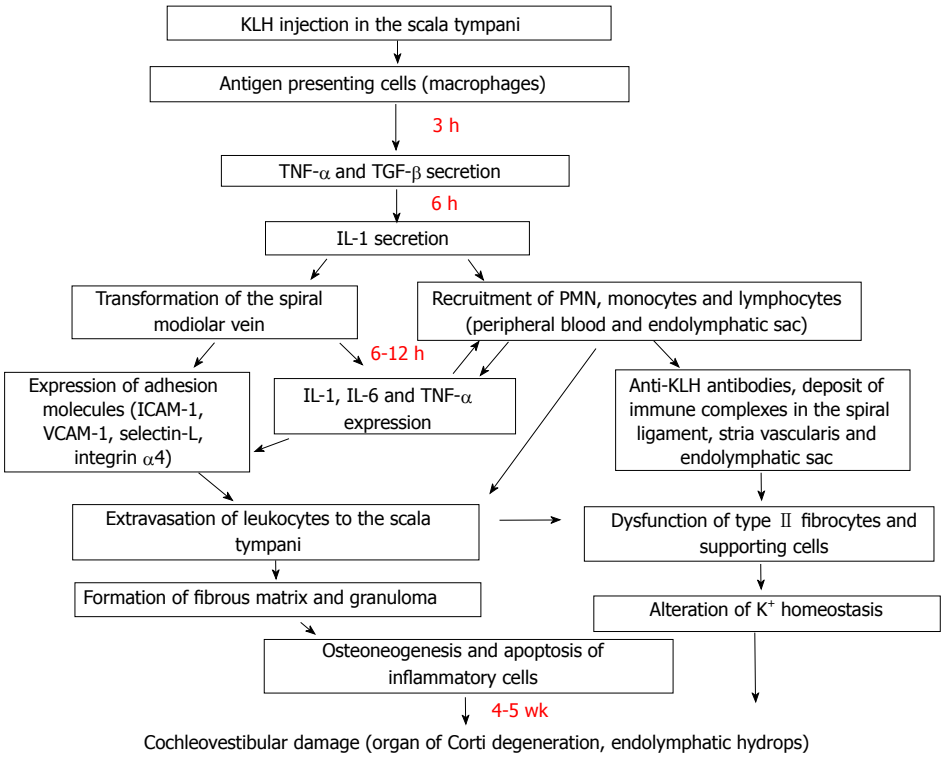

Figure 1 Immunopathological sequence in experimental labyrinthitis by keyhole limpet hemocyanin. KLH: Keyhole limpet hemocyanin; TNF-α: Tumor necrosis factor alpha; TGF-β: Transforming growth factor beta; IL-1: Interleukin 1; PMN: Polimorphonuclear cells; ICAM-1: Intercellular adhesion molecule 1; VCAM-1: Vascular cellular adhesion molecule 1.

CLINICAL PRESENTATION

AIED can present alone or associated with other autoimmune systemic disease (secondary AIED). Generally, three forms of clinical presentation are recognized: sudden deafness, rapidly progressive hearing loss, and fluctuating hearing loss. Vertigo appears in almost 50% of cases, making AIED difficult to distinguish from Meniere's disease[17]. Eventually, AIED can affect both ears and progress to deafness unless a correct diagnosis is made and prompt treatment is established. Since this clinical picture is not specific, and could cover almost all inner ear disorders, diagnostic criteria are needed to orientate the introduction of medical treatment.

DIAGNOSIS

A prompt diagnosis and treatment has a great impact on the hearing prognosis of patients with AIED and thus has stimulated the search for specific markers of inner ear inflammation. The presence of autoantibodies is usually the first step in recognizing the autoimmune nature of a disease, but this is not enough as autoantibodies are also common in people without an autoimmune disease. In fact, autoantibodies against specific cochlear antigens have a low specificity for rapidly progressive sensorineural hearing loss. Although other localized autoimmune diseases such as pemphigus or cutaneous vasculitis are diagnosed by immunofluorescence in biopsied tissues, this is not possible in the case of AIED because an inner ear biopsy represents the destruction of the organ and its function.

The diagnosis of AIED is based fundamentally on clinical evaluation, the demonstration of a progressive sensorineural hearing loss in periodic audiological tests and a response to immunomodulatory drugs such as corticosteroids.

Once other causes of sensorineural hearing loss have been ruled out, patients undergo a battery of non-specific test common to other autoimmune diseases: blood tests, biochemistry, erythrocyte sedimentation rate, etc. In certain cases, serological studies can detect some specific autoantibodies, but there are no immunological or serological tests that are specific or sensitive enough to establish a definitive diagnosis[18]. A summary of the main specific and non-specific autoantibodies found in AIED patients and experimental models is given in Table 1. Most studies deal with one or several autoantibodies and no single study has examined them all in the same population.

Immunological tests

Numerous tests have been proposed, antibodies against collagen type II, endothelial cells, sulfoglucoronosyl glycolipids, major peripheral protein P0, *etc* (Table 1). The role of immune complexes and changes in blood lymphocytic populations has also been studied[19].

Western blot

Harris and Sharp proposed this technique to identify spe-

Table 1 Specific and non-specific autoantibodies present in autoimmune inner ear disease patients and/or animal models of sterile labyrinthitis

Specific autoantibodies
Collagen type Ⅱ
Collagen type IX
Cochlin
DEP-1/CD 148
KHRI-3
Myelin protein P0
Raf-1
Beta-tectorin
Beta-actin
Connexin 26
Non-specific autoantibodies
Antinuclear antibodies
Anti-neutrophil cytoplasmic antibodies
Anti-endothelial cell antibodies
Rheumatoid factor
Heat shock protein of 70 kDa
Anti-phospholipids/anticardiolipin antibodies
Antithyroid antibodies

cific autoantibodies against inner ear antigens in immunized animals and in patients with sensorineural hearing loss[20]. Among these autoantibodies, the most studied is an antibody that binds to a 68 kD antigen derived from a bovine temporal bone extract and the inducible form of heat shock protein 70 (HSP-70)[21]. HSP-70 is expressed in a variety of pathological inner ear conditions, as a marker of early cell damage, but is not specific. In AIED patients, the frequency of antibodies against HSP-70 is not different in patients and controls and is not useful in the diagnosis of AIED[22]. Mice immunized with HSP-70 produce anti HSP-70 antibodies without presenting a hearing loss, which indicates that these antibodies are not directly involved in the pathogenesis of AIED. However, these antibodies could have a role as markers of disease activity and treatment response.

Imaging studies

Neither magnetic resonance imaging (MRI) nor positron emission tomography (PET) has demonstrated their utility in the diagnosis of AIED in spite of early promising results.

Diagnostic profiles

Although specific tests have an unquestionable value, there is no currently available test that has proved to be effective. For this reason, the development of diagnostic profiles can contribute to cost saving by restricting the diagnostic tests to those which are really cost-effective[23]. A proposed diagnostic profile for AIED is shown in Table 2. An AIED is suspected when three major criteria or two major and two minor criteria are met.

Table 2 Diagnostic profile for autoimmune inner ear disease

Major criteria
Bilateral hearing loss
Systemic autoimmune disease
ANA > 1:80
Decrease of native T cells (CD4CD45RA)
Hearing recovery rate > 80%
Minor criteria
Unilateral hearing loss
Young or middle aged
Woman
Hearing recovery rate < 80%

Hearing recovery rate (after immunosuppressive therapy) is obtained: (initial hearing levels - final hearing levels)/initial hearing level – opposite ear hearing levels) × 100 (%). An autoimmune inner ear disease is suspected when three major criteria or two major and two minor criteria are met.

TREATMENT

The treatment most widely used for AIED is corticosteroids therapy. The initial dosage regimen is 60 mg or 1 mg/kg per day of prednisone or 6-methylprednisolone for a month. Shorter courses or lower doses have proved to be ineffective and increase the risk of relapse[24]. In rapidly progressive forms 1 mg/kg per day is maintained for 4 wk until the audiogram is stable and the dose is then tapered over 8 wk to 10-20 mg per day, which is maintained for another 6 wk. In cases of sudden hearing loss, 1 mg/kg per day of 6-methylprednisolone is administered for four weeks. In severe hearing loss (over 70 dB) three pulses of 500 mg are administered, and then the above-mentioned dosage regimen is applied. When patients receive high doses of corticosteroids, active tuberculosis must be ruled out, and glycemia, potassium and blood pressure must be monitored. Tapering must be gradual, slower if glucocorticoids have been given at higher doses or for a longer time.

In AIED patients, severe adverse reactions have rarely been reported (0%-0.9%) though they may be more frequent when high dose intravenous pulse corticosteroids are employed. The overall rate of side effects is not greater than 7.8%[25].

Other immunosuppressants

Some patients do not respond to corticoids or require high doses to control the disease, and other immunosuppressants such as methotrexate or cyclophosphamide have been tried. The empirical basis for using these drugs is the observation that in certain cases their effect enhances that of the corticosteroids, thus obtaining remission of one or more symptoms that is not achieved with corticosteroids alone, or allowing reduction of the required dose of corticosteroids to maintain the patient symptom-free.

Methotrexate: A meta-analysis showed that there was no benefit with methotrexate compared with corticosteroids alone[26]. However, vertigo or instability can improve with long treatments.

The most frequently employed regimen is 7.5 mg weekly administered in one single dose. Once the response is achieved, the drug is given orally (15 mg weekly) for 12 mo. Methotrexate is associated with blood toxicity (leukopenia, thrombocytopenia), liver toxicity

Table 3 Biological therapy agents

Drug	Anti	Dosage	Licensed indications	EMA approval	FDA approval
Etanercept	TNF-α	25 mg × 2/wk or 50 mg/wk *sc*	RA, JRA, PsA, AS, Ps	2000	1998
Infliximab	TNF-α	3 mg/kg at 0, 2 and 6 wk followed by 3.5-7.5 mg/8 wk *iv*	RA, PsA, AS, Ps, UC, CD	1999	1998
Adalimumab	TNF-α	40 mg/1-2 wk *sc*	RA, JRA, PsA, AS, Ps, CD	2003	2002
Anakinra	IL-1	100 mg/d *sc*	NHL, CLL, RA	2002	2001
Rituximab	B-cell CD20R	1 g/wk × 2 *iv*	RA	1998	1997

Dosage and indications approved by the European Medicines Agency (EMA) and the United States Food and Drug Administration (FDA). RA: Rheumatoid arthritis; JRA: Juvenile rheumatoid arthritis; PsA: Psoriatic arthritis; AS: Ankylosing spondylitis; Ps: Psoriasis; UC: Ulcerative colitis; CD: Crohn´s disease; NHL: Non-Hodgkin lymphoma; CLL: Chronic lymphocytic leukemia.

(elevated liver enzymes, periportal fibrosis, cirrhosis) and gastrointestinal toxicity (nausea, vomiting, mucositis). Folic acid supplements reduce the adverse effects, preserve its efficacy and are, therefore, recommended.

Cyclophosphamide: This drug was used by McCabe[1], who advocated its use as the treatment of choice, in his original series of cases. However, because of its adverse effect profile (gonadal, bladder and bone marrow toxicity) it is not frequently used and is limited to those patients who do not respond to corticosteroids or do not maintain their response after dose tapering. The oral dose is 1-2 mg/kg per day for 4-6 wk. Intravenously, the starting dose is 0.75 g/m^2 or 0.5 g/m^2 if the glomerular filtration rate is lower than a third of the normal value, and this is repeated every 1-3 mo. The white cell count should not be lower than 2000/mm^3 and neutrophils should remain over 1000/mm^3. When both cyclophosphamide and high doses of corticosteroids are employed trimethoprim/sulphamethoxazole or dapsone is administered to prevent *Pneumocystis carinii* pneumonia.

Plasmapheresis

This procedure allows the blood to be separated into its two components - blood cells and plasma - and allows some components such as antibodies to be removed before the cells and plasma are transfused back to the patient. In a long term study performed in AIED, 50% of the patients achieved an improvement or stabilization of hearing loss after this therapy[27].

NOVELTIES IN AIED THERAPY

The overall response rate to corticosteroids is 60%, but the response rate varies considerably. In most responders the dose can be lowered or corticosteroids can be withdrawn without relapse, but some patients can present a corticosteroid-dependant hearing loss. Hearing loss may become refractory to corticosteroids, and other immunosuppressants should be considered in these cases. Finally, treatment can result in unacceptable adverse reactions (gastritis, peptic ulcer, fluid retention, glucose intolerance, avascular necrosis of the femoral head, psychiatric problems, sleep disorders, cataracts, osteoporosis, cushingoid habitus) and this has prompted the search for new drugs or different modes of administration such as the intratympanic route.

Intratympanic therapy

The use of intratympanic corticosteroids is an attractive therapeutic approach because it is minimally invasive and, since the drug is applied directly to the affected ear, side effects are minimized. However, there is no consensus regarding the doses and length of treatment. Moreover, it is not easy to control the dose that actually enters the inner ear (part of it is absorbed in the middle ear and part is eliminated through the Eustachian tube); as a result, its efficacy has so far not been fully determined[28].

Biological therapy agents

Biological therapy agents are fusion proteins (made from a fusion gene, which is created by joining parts of two or more genes) or monoclonal antibodies designed to block specific components of the inflammatory cascade. Tumor necrosis factor α inhibitors and lymphocyte CD20 receptor antagonists have recently been tested on AIED patients (Table 3).

Among the biological therapy agents the most frequently used are tumor necrosis factor alpha blockers. Tumor necrosis factor (TNF) is a proinflammatory cytokine produced by multiple cells, especially macrophages, that stimulates the maturation and migration of dendritic cells, activates neutrophils and NK cells, and increases vascular permeability. It was isolated by Carswell *et al*[29] in 1975 when they were seeking to identify the factors responsible for Meth A sarcoma necrosis. It is expressed early in the inflammatory response in different inner ear structures. Of the different TNF-α blockers that have been developed, etanercept, infliximab and adalimumab have been tested on AIED patients. X-ray or Mantoux screening is recommended before initiating treatment with TNF-α blockers because TNF-α is a key component in the body's defense against *M. tuberculosis* and other granulomatous diseases.

Etanercept: The results obtained so far are promising but not conclusive, as very few studies have been performed[30]. Anecdotically, it has been used together with methotrexate with good results, allowing corticosteroid therapy to be withdrawn[31]. The usual dose is 25 mg administered by subcutaneous injection twice a week or 50 mg once a week for an indefinite period of time. Side

effects that have been a concern are infections including tuberculosis and sepsis, tumors such as lymphomas, anemia and pancytopenia, demyelinating diseases, congestive heart failure and hypersensitivity. However, a meta-analysis that examined the adverse reactions with etanercept and other biologic therapies in 163 randomized controlled studies with 50010 participants and 46 extension studies with 11954 participants reported that the severe adverse reactions rate for the biological products was not different from that of the control therapy (*e.g.*, corticosteroids)[32].

Adalimumab: It is administered by a subcutaneous injection of 40 mg every two weeks for an indefinite period of time. The dose can be increased to 40 mg weekly if a decrease in the response is observed. It has been employed successfully in one patient with autoimmune sensorineural hearing loss and rheumatoid arthritis[33].

Infliximab: The usual regimen is slow intravenous infusion (2 h) of 3 mg/kg at the start of treatment, and at 2 and 6 wk, followed by maintenance therapy every 8 wk indefinitely. Intratympanic administration of infliximab can help to reduce corticosteroids doses in patients with AIED[34].

B lymphocyte CD20 receptor antagonist: Apart from TNF-α blockers other biological therapy agents such as rituximab have been recently tested on patients with AIED. Rituximab is a chimeric monoclonal antibody that binds to the CD20 receptor of B lymphocytes, thereby inducing apoptosis and reducing their number. The few studies that have used rituximab in AIED patients have yielded encouraging results[35,36]. However, more studies are needed for reliable conclusions to be reached. The recommended dose is 1000 mg in intravenous injection, followed by a second injection perfusion of 1000 mg 2 wk later. The most common side effect associated with rituximab is a reaction to the injection (low blood pressure, nausea, eruption, fever, itching, urticaria, throat irritation, tachycardia, peripheral edema). Infections of the upper airway and urinary tract have also been reported (but not in AIED patients).

Future possibilities in AIED therapy

Anakinra is an IL-1 inhibitor that has been successfully used in chronic infantile neurological cutaneous and articular (CINCA) syndrome and Muckle-Wells syndrome, which can present with hearing loss and belong to a group of autoinflammatory febrile syndromes caused by mutations in the CIAS/NALP3 gene on chromosome 1q4432[37]. These mutations seem to interrupt apoptosis mechanisms and lead to overexpression of IL-1 with devastating proinflammatory effects. The role that anakinra could have in the management of AIED has yet to be elucidated (Table 3).

Other lines of AIED therapy not available at present are represented by gene therapy and stem cell therapy, which would attempt to repair established damage to the inner ear. These therapeutic strategies are based on the knowledge of cell signaling routes involved in the development of the cochlear sensorial epithelium during embryogenesis. This sensorial epithelium derives from a group of cells that after several divisions start to differentiate into hair cells and supporting cells. Adult mammals have lost the capacity to regenerate damaged hair cells[38]. Gene and stem cell therapy attempt to revert this situation[39]. However, both approaches present the same hazards and difficulties: access to the whole cochlea, integration and maturation of hair cells in the correct position within the cochlea and not in ectopic locations, and risk of tumor development[40]. At present, these difficulties need to be overcome before clinical trials can be started.

CONCLUSION

Different animal models of experimental labyrinthitis have contributed to the understanding of AIED pathophysiology. In particular, the model of experimental labyrinthitis by KLH has allowed a chronological sequence of inner ear damage to be established and has, therefore, provided a rational basis for testing new therapies.

In spite of all the efforts to find a good marker for the disease, the available tests are not specific or sensitive enough to establish a definitive diagnosis. However, the search for specific autoantibodies for AIED remains a valid approach, because the diagnostic value of autoantibodies depends on a statistical and epidemiological association with disease more than on a cause-effect relation. It would be very useful to study how these autoantibody titers vary with time and with response to therapy. Moreover, some autoantibodies could provide information on group of patients with different prognoses or different clinical responses.

Finally, new treatments have been tested recently. Biologics, a new family of immunomodulatory agents, could play a role in the treatment of AIED in the future, and the first studies conducted with these drugs have produced promising results. They could be indicated in patients who do not respond to, or who have become refractory to, glucocorticoids. However, more clinical studies are necessary to evaluate their real value. Intratympanic therapy avoids many of the adverse reactions associated with currently used drugs, but this approach has not been sufficiently evaluated yet.

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REVIEW

Adult stem cell-based apexogenesis

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Abstract

Generally, the dental pulp needs to be removed when it is infected, and root canal therapy (RCT) is usually required in which infected dental pulp is replaced with inorganic materials (paste and gutta percha). This treatment approach ultimately brings about a dead tooth. However, pulp vitality is extremely important to the tooth itself, since it provides nutrition and acts as a biosensor to detect the potential pathogenic stimuli. Despite the reported clinical success rate, RCT-treated teeth are destined to be devitalized, brittle and susceptible to postoperative fracture. Recently, the advances and achievements in the field of stem cell biology and regenerative medicine have inspired novel biological approaches to apexogenesis in young patients suffering from pulpitis or periapical periodontitis. This review mainly focuses on the benchtop and clinical regeneration of root apex mediated by adult stem cells. Moreover, current strategies for infected pulp therapy are also discussed here.

Key words: Apexogenesis; Dental pulp; Stem cell; Odontoblast; Tooth regeneration

Core tip: Compared with traditional root canal therapy, stem cell-based therapies initiate a new approach to treating dental pulp diseases. The development of teeth depends on many kinds of stem cells and some of which still exist after the formation of the root, creating a chance for the tooth to regenerate itself when it stops developing due to infection or trauma. This article provides an interesting view on the benchtop and clinical regeneration of root apex mediated by adult stem cells. Moreover, current strategies for infected pulp therapy are also discussed.

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INTRODUCTION

When the dental pulp is infected, traditionally, the dental pulp must be replaced with inorganic materials (paste and gutta percha) *via* root canal therapy (RCT). However, for RCT-treated teeth, the loss of pulp vitality, which primarily provides nutrition and acts as a biosensor to detect the potential pathogenic stimuli, will bring about various problems including the decreased strength and increased fragility; teeth are destined to be dead, devitalized, brittle and susceptible to postoperative fracture. Therefore, crowns are suggested to protect the non-vital tooth, but

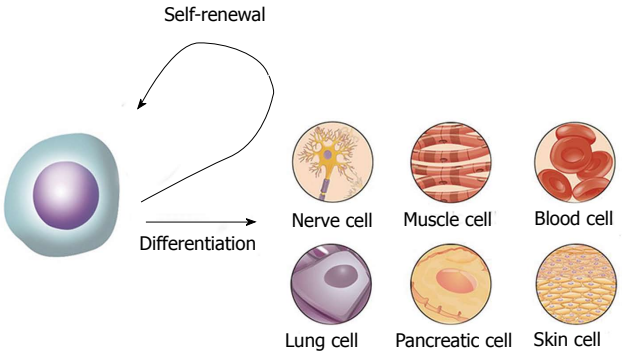

Figure 1 Stem cells have the capacity of self-renewal and multiple-differentiation.

subsequently bring about other complications, including food impaction, recurrent caries, gingivitis, coronal leakage or microleakage, *etc.*

As a result, a better way to treat dental pulp diseases is needed. Nowadays stem cell-based therapies represent a promising potential to improve the life of patients with conditions ranging from neurodegenerative and traumatic diseases to regenerative disorders requiring replacement of complex structures such as bones and teeth[1-3]. Stem cells have the capacity of self-renewal and multiple-differentiation (Figure 1). They can be divided into four types, including totipotent stem cells, pluripotent stem cells, multipotent stem cells and unipotent or progenitor stem cells[2]. Due to the ethical and legal issues, the clinical application of embryonic stem cells is still controversial and restricted, although they can differentiate into almost every cell type in the human body[1]. Adult stem cells, however, become valuable because they can be isolated from many different adult tissues and demonstrate the potential to give rise to cells of various lineages[4]. The typical adult stem cells, like neural stem cells (NSCs), hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs), are considered as multipotent because they can give birth to different cell types in the tissue of origin[5]. NSCs are present in the subventricular zone and the subgranular zone of the hippocampal dentate gyrus and are able to generate three cell types of the brain including neurons, astrocytes, and oligodendrocytes. HSCs were first named by Cohneim about 130 years ago[6]. They can be isolated from bone marrow and differentiate into all blood cells of the myeloid and lymphoid cell lineages[3,7]. HSCs are the best-studied stem cells for transplantation. They have been used in stem cell-based therapies for decades, especially in an allogeneic setting[8]. Hematopoietic disorders or patients with other malignancies undergoing intensive chemotherapy or radiation therapy are usually treated with this kind of stem cells[9]. Another kind of adult stem cells were first described by Friedenstein in the 1970s and were later defined as "MSCs" by Caplan and others[10-13]. MSCs have the potential to differentiate towards lineages of mesenchymal origin, including bone, cartilage, fat, connective tissue, muscle and marrow stroma. They can be isolated from diverse organs and tissues, such as bone marrow, adipose tissue, umbilical cord blood and stroma, placenta, amniotic membrane, synovium, lung, dental pulp tissue and so on[3,5]. Recently, more attention was paid to the clinical use of MSC transplantation to treat diseases that affect the host organ, including kidney injury, liver failure, myocardial infarction, articular cartilage defect and spinal cord injury, *etc.*[14-18]. However, the application in the treatment of a young infected tooth remains unclear.

During tooth development, enamel is formed by ameloblasts derived from the oral epithelium, while dentin and dental pulp originate from the dental papilla. The development of tooth depends on many kinds of stem cells, some of which still exist after the formation of the root. It offers a chance for the tooth to regenerate itself when the tooth stops developing because of infection or trauma. Generally, apexification by calcium hydroxide (CH) is the most traditional method to treat the immature tooth suffering from infection, usually bringing about a calcified barrier in the root with an open apex or an incompletely formed root with necrotic pulp[19,20]. However, the root often stops developing, leading to an imbalanced crown and root containing thin root dentin and wide apex, which may cause root fracture during functional movements, and it also takes a long period to complete the treatment. Recently, another method called apexogenesis has been developed with the emergence of mineral trioxide aggregate (MTA), which refers to a vital pulp therapy procedure that encourages physiological development and formation of the root end[21,22]. Compared to apexification, teeth after apexogenesis with vital pulp therapy develop a normal thickness of dentin, root length and apical morphology with fewer follow-up appointments[23,24]. In cases of Jung *et al*[25], he observed a separately growing root after the apexification treatment, which indicated the coexistence of apexification and apexogenesis. It is believed that some stem cells play important roles in the continuing root development of infected immature tooth.

ADULT STEM CELL CANDIDATES

MSCs with the capacity of self-renewal and multi-lineage differentiation are regarded as attractive progenitor cell sources for tissue engineering and regeneration[26]. To date, several kinds of MSCs have been identified as promising candidates for dental tissue engineering in the dentistry field such as bone marrow MSCs (BMMSCs), dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHEDs), periodontal ligament stem cells (PDLSCs), dental follicle precursor cells (DFPCs) and stem cells from apical papilla (SCAPs)[26-30]. Which MSCs are mostly suitable for apexogenesis? On one hand, a tooth derives from a tooth germ consisting of various MSCs which can develop certain parts of the tooth. On the other hand, MSCs of different origins may present various differentiation abilities. Previous studies have revealed that MSCs can differentiate into

Table 1 Adult stem cell candidates

	BMMSCs	DPSCs	SCAPs	SHEDs	PDLSCs	DFPCs
Location	Bone marrow	Permanent tooth pulp	Apical papilla of developing root	Exfoliated deciduous tooth pulp	Periodontal ligament	Dental follicle
Specific markers	ND	ND	CD24	ND	ND	GoPro49
Proliferation rate	Moderate	High	High	High	High	High
Multi-potentiality	Odontoblasts, osteoblasts, adipocytes, chondrocytes, myocytes, neurocytes, tenocytes	Odontoblasts, osteoblasts, adipocytes, chondrocytes, myocytes, neurocytes	Odontoblasts, osteoblasts, adipocytes, chondrocytes, neurocytes	Odontoblasts, osteoblasts, adipocytes, chondrocytes, myocytes, neurocytes	Osteoblasts, chondrocytes, adipocytes, neurocytes, cementoblasts	Osteoblasts, chondrocytes, cementoblasts, adipocytes, PDL fibroblasts, neuron-like cells
In vivo transplantation	Dentin-pulp-like tissue, bone, cementum, PDL	Dentin-pulp complex, bone	Dentin-pulp-like complex, bone	Dentin-like tissue, bone	Cementum/PDL-like structure	Cementum-like structure, bone

ND: Not determined; BMMSCs: Bone marrow mesenchymal stem cells; DPSCs: Dental pulp stem cells; SCAPs: Stem cells from apical papilla; SHEDs: Stem cells from human exfoliated deciduous teeth; PDL: Periodontal ligament; PDLSCs: Periodontal ligament stem cells; DFPCs: Dental follicle precursor cells.

Table 2 Comparison of *in vivo* transplantation outcomes of mesenchymal stem cells from different researchers

Ref.	MSCs	Outcomes
Ohazama *et al*[42]	BMMSCs	Tooth structures and associated bone
Li *et al*[28]	BMMSCs	Tooth-like structures surrounded by bone and soft tissues
Kawaguchi *et al*[43]	BMMSCs	Histologically produced cementum, PDL and alveolar bone
Gronthos *et al*[44]	DPSCs	Pulp-dentine like tissue complexes lined with odontoblast-like cells
Carinci *et al*[45]	DPSCs	Bone-like tissues
Sonoyama *et al*[49]	SCAPs	A typical dentin structure with connective tissues
Battula *et al*[39]	SCAPs	Bone-/dentin-like mineralized tissues
Miura *et al*[53]	SHEDs	Dentin-like tissue, but not a dentin-pulp-like complex; not differentiate directly into osteoblasts but only induce new bone formation
Wang *et al*[55]	SHEDs	Exhibit an enhanced potential to form bone
Park *et al*[57]/Seo *et al*[58]	PDLSCs	A typical cementum/PDL-like structure
Morsczeck *et al*[64]	DFPCs	A structure consists of fibrous or rigid tissue, no dentin, cementum, or bone formation
Handa *et al*[65]	DFPCs	Cementum-like matrix
Honda *et al*[66]	DFPCs	Bone

MSCs: Mesenchymal stem cells; SHEDs: Stem cells from human exfoliated deciduous teeth; DPSCs: Dental pulp stem cells; DFPCs: Dental follicle precursor cells; PDLSCs: Periodontal ligament stem cells; SCAPs: Stem cells from apical papilla; BMMSCs: Bone marrow MSCs; PDL: Periodontal ligament.

certain tissues after specific induction. Accumulating evidence demonstrates that MSCs have the potential to cross lineage boundaries, even able to differentiate into specific cells of tissues beyond their origin[31] (Tables 1 and 2 describe the adult stem cell candidates and their *in vivo* transplantation outcomes from different research groups).

BMMSCs

BMMSCs are the first isolated MSCs with a spindle-shaped morphology which have the ability to adhere to a plastic surface with high proliferative potential[32]. BMMSCs possess the self-renewal capacity to form colonies *in vitro* and are capable of differentiating into multiple mesenchymal cell lineages such as osteoblasts, adipocytes, chondrocytes, muscle cells, tenocytes, and nerve cells[33-35]. However, BMMSCs are limited to a growth potential of 30 to approximately 50 population-doublings (PDs) following *ex vivo* expansion[30]. BMMSCs express the Oct-4, Nanog, STRO-1, CD73, CD90, CD105, CD146[36,37] and are negative for CD14, CD34, CD45 and human leukocyte antigen-DR[38-40]. Based on their multi-lineage differentiation potential and their high proliferative capacity, BMMSCs have a great potential for stem cell-based regenerative therapies. For instance, the intracoronary transplantation of autologous BMMSCs for ischemic cardiomyopathy has shown the promising results[41]. Furthermore, after transplantation of BMMSCs into regions of central nervous injury, an improved functional recovery was observed in the injured rodent brain or spinal cord[3]. Ohazama *et al*[42] have reported that the combination of adult BMMSCs and embryonic oral epithelium can stimulate an odontogenic response in BMMSCs, and transfer of the complex into adult renal capsules can result in the development of tooth structures and associated bone. Li *et al*[28] also have demonstrated that the combination of oral epithelial cells from rat embryos with BMSSCs can generate tooth-like structures expressing dentin sialophosphoprotein (DSPP) and dentine matrix protein 1 (DMP1) surrounded by bone and soft tissue. Kawaguchi *et al*[43] have shown complete regeneration of periodontal defects after BMMSC

transplantation, and histologically produced cementum, periodontal ligament (PDL), and alveolar bone.

DPSCs

DPSCs were first isolated and characterized from dental pulp tissue by Gronthos *et al*[44] in 2000. Similar to MSCs, DPSCs are positive for CD29, CD44, CD59, CD90, CD106, and CD146, and negative for CD34, CD45, and CD11b. DPSCs are described as a highly proliferative cell population with the self-renewal ability and multi-lineage differentiation potential[3]. DPSCs possess mesenchymal stem cell properties such as a fibroblast-like morphology, adherence to a plastic surface, and the ability to form colonies when cultured *in vitro*[3] and they are able to differentiate into chondrocytes, adipocytes, odontoblasts and neural-like cells under appropriate inductive conditions[2]. Previous studies have shown that DPSCs are capable of differentiating into odontoblastic lineages *in vitro* and form ectopic pulp-dentine like tissue complexes lined with odontoblast-like cells expressing DSPP when transplanted subcutaneously into immunocompromised mice *in vivo*[44]. DPSCs can also form bone-like tissues when transplanted into immunocompromised mice[45]. Some studies have demonstrated that DPSCs are able to differentiate into endothelial-like cells and express blood vessel markers and neural markers, but the *in vivo* differentiation potential is still under debate[46-48]. Due to their easy obtainment and the potential of multi-lineage differentiation, DPSCs are thought to be an ideal cell source for tissue regeneration and engineering.

Stem cells from apical papilla

Apical papilla means the soft tissue at the apices of developing permanent teeth[49] and stem cells from apical papilla (SCAPs) are a population of MSCs residing in the apical papilla of incompletely developed teeth[50]. The surface makers are similar to those of BMMSCs and DPSCs, but CD24 is only detected in SCAPs. The expression of CD24 is down-regulated following osteogenic induction. It has been reported that SCAPs display a higher proliferation rate than DPSCs, probably because they are derived from a developing tissue[51]. Similar to DPSCs, SCAPs are able to differentiate into a variety of cell types, but appear to have greater dentinogenic potential than DPSCs. An *in vivo* study has shown that SCAPs with hydroxyapatite/tricalcium phosphate particles that were transplanted into immunocompromised mice can generate a typical dentin structure with a layer of dentin tissue formed on the surface of the hydroxyapatite/tricalcium phosphate along with connective tissue[49]. SCAPs also demonstrate the capacity to undergo adipogenic differentiation after induction and express neural markers with or without stimulation when cultured *in vitro*[30]. Besides, SCAPs are able to form a bio-root with the use in combination with PDLSCs[52]. All the above findings suggest that SCAPs can be used for tissue regeneration and engineering. Based on previous findings, SCAPs appear to be the source of primary odontoblasts that are responsible for the formation of root dentin[49]. This may explain the clinic phenomenon that apexogenesis can occur in infected immature permanent teeth suffering from apical periodontitis or abscess[23,51].

Stem cells from human exfoliated deciduous teeth

Stem cells from human exfoliated deciduous teeth (SHEDs) were first isolated by Miura *et al*[53] from dental pulp tissue derived from exfoliated deciduous teeth. SHEDs exhibit a higher proliferation rate than DPSCs. They express similar surface markers as compared to DPSCs and BMMSCs, but the expression of CD105 and CD146 is higher, suggesting higher capacity for differentiation. SHEDs have the potential to differentiate into neurons, adipocytes, osteoblasts, and odontoblasts as well as DPSCs[54]. When SHEDs are subcutaneously transplanted into immunocompromised mice, they can form ectopic dentin-like tissue, but are not able to regenerate a dentin-pulp-like complex[2]. Wang *et al*[55] have revealed that SHEDs exhibit an enhanced potential to form bone, while Miura *et al*[53] have suggested that SHEDs can not differentiate directly into osteoblasts but only induce new bone formation. These findings imply that deciduous teeth may be involved in bone formation during the eruption of permanent teeth. SHEDs can also differentiate into neural cells after neuronal induction and express early neuronal markers. As a result, SHEDs can be a promising source of stem cells for regenerative medicine.

PDL stem cells

PDL is the soft connective tissue interposed between the cementum and the inner wall of the alveolar socket[56], which is derived from the dental follicle. Previous studies have suggested that the PDL space may contain stem cells that exhibit osteogenic, cementoblastic, adipogenic, chondrogenic and neurogenic characteristics under certain culture conditions[30,57], which are defined as PDL stem cells (PDLSCs). When cultured *in vitro*, PDLSCs can form mineralized nodules, express the bone-associated markers including alkaline phosphatase and bone sialoprotein (BSP) in response to bone-inductive factors such as insulin-like growth factor 1 and express high level of scleraxis, which is a specific transcription factor associated with tendon cells[58,59]. Although PDLSCs express a range of cementoblastic/osteoblastic markers, they do not form dentin and its associated haemopoietic components *in vivo*. Previous studies have demonstrated that PDLSCs transplanted into immunocompromised mice can generate a typical cementum/PDL-like structure, in which a thin layer of cementum-like tissue is formed on the surface of the carrier, along with condensed collagen fibres with sparse cells that resemble PDL structures[57,58]. But the cemetum/PDL-like structures appeared totally different from typical bone/marrow structures generated by BMMSCs and dentin/pulp-like structures generated by DPSCs[58]. Due to their capacity to form periodontal structures, PDLSCs can be used as a cell source for the treatment of periodontal diseases, tissue engineering and

stem cell-based therapies.

Dental follicle precursor cells

Dental follicle is an ectomesenchymal tissue that surrounds the enamel organ and the dental papilla of the developing tooth germ prior to eruption. This tissue contains progenitor cells that form the periodontium including cementum, PDL, and alveolar bone. Dental follicle precursor cells (DFPCs) are isolated from human dental follicles of impacted third molars, expressing typical mesenchymal stem cell markers such as STRO-1, CD13, CD44, CD73, Notch1, and nestin[60,61]. Moreover, GoPro49, a novel Golgi protein, has been identified as a specific marker for DFPCs[62]. DFPCs possess the ability to differentiate into osteoblasts/cementoblasts, chondrocytes, adipocytes, and neuron-like cells when growing under the appropriate culture conditions *in vitro*[3,60,61]. Recent studies have revealed that DFPCs cultured at 38 to 40 ℃ demonstrate greater osteogenesis, indicating that appropriate heat-stress treatments can promote their differentiation[63]. When DFPCs were transplanted into immunocompromised mice, a structure comprised of fibrous or rigid tissue was generated, expressing BSP, osteocalcin (OCN) and collagen type I [64]. However, there were no dentin, cementum, or bone formation observed in the transplants *in vivo*. But some studies[65,66] have demonstrated that DFPCs can form cementum-like matrix or bone structures in the subcutaneous area of immunodeficient mice. More work still has to be performed to explore their potential capability during the cellular therapies for periodontal diseases.

PUTATIVE INDUCTIVE MATERIALS

In addition to stem cells, adequate inductive materials that can promote the differentiation of stem cells are essential to the success of stem cell-based treatment. Previous studies have investigated the effects of several materials on different stem cells, which may guide us to pair the right stem cells with the most compatible inductive material.

CH

CH (pH = 12.5) has a good antimicrobial characteristic and can inhibit tooth resorption and induce the hard tissue formation[67]. It has been successfully utilized in various endodontic treatments, such as apexification, apexogenesis, pulp capping, pulpotomy, and routine root canal therapy in infected canals for its potential to induce hard tissue repair at the site of pulp exposure[68,69], An earlier study has suggested osteo-inductive properties of CH[70], however, no significant changes were observed *in vitro*[71]. When CH is placed at the exposed pulp site, damaged primary odontoblasts are replaced with newly differentiated odontoblast-like cells. Ji *et al*[68] have exhibited that CH can increase the recruitment, migration, proliferation, and mineralization of DPSCs and PDLSCs. Besides, Ruparel's work has suggested that CH can promote survival of SCAPs[72]. Moreover, low concentrated CH induces the proliferation of pulp fibroblasts. However, there exist several disadvantages of CH used in apexification, including multiple visits, the long treatment time and the risk of root fracture as a result of long-term use of CH[67,73-75]. It is also suggested that direct contact of CH with the tissue will induce the formation of calcified tissue in the pulp space, thus preventing pulp tissue from regeneration[23]. Another problem is that CH may damage the Hertwig's epithelial root sheath and thereby destroy its ability to induce the nearby undifferentiated cells to become odontoblasts[73].

Mineral trioxide aggregate

In 1993, mineral trioxide aggregate (MTA) was firstly introduced into endodontics and now has been widely used in diverse endodontic therapies, including pulp capping, pulpotomy, apical barrier formation, apexogenesis in developing teeth, repair of root perforations and root canal filling. MTA is a cement mixture that consists of different oxide compounds, including sodium and potassium oxides, calcium oxide, silicon oxide, ferric oxide, aluminum oxide, and magnesium oxide[76]. Compared with CH, MTA is a better choice for direct pulp capping because of its lower solubility, improved mechanical strength, better marginal adaptation, and better sealing ability, but no significant histological difference is established[77,78]. Some data also suggest that MTA is more predictable with consistent hard-tissue formation[79] as a result of the release of a large number of Ca^{2+} ions or the secretion of bone morphogenetic protein 2 and transforming growth factor-beta 1 by periodontal fibroblasts[80]. Some studies have reported that MTA can induce the formation of hard tissue in a shorter period of time than CH[81,82]. Further study revealed that MTA can induce tissue regeneration *via* the promotion of mesenchymal stem cell adhesion, proliferation, and migration[83]. Recent studies have shown that MTA stimulates the odontogenic differentiation of DPSCs, with the up-regulation of OCN and DSP[84-86]. The significantly increased levels of the angiogenic factors such as vascular endothelial growth factor and fibroblastic growth factor-2 of DPSCs are observed following the treatment with MTA *in vitro*[84,85], which play a critical role in tissue development, cell migration, inflammation and wound repair.

Dentine

Generation of well-vascularized pulp-like tissue by using a tooth slice model has been reported. Huang *et al*[30] suggested that DPSCs differentiate into odontoblast-like cells with a cellular process extending into dentinal tubules when seeded onto the existing dentine. Cordeiro *et al*[87] have demonstrated similar findings that odontoblast-like cells arose from the stem cells and localized against the existing dentine surface in their *in vivo* study model. Previous studies have shown that human DPSCs, SCAPs and SHEDs, in combination with synthetic scaffolds or human root seg-

ments, are able to generate vascularized pulp-like tissues and form dentin-like mineral structures depositing onto the existing dentinal wall in the root canal space[88-90]. The mechanism behind this phenomenon has been speculated to be related with the released growth factors by dentine, such as TGF-β, which attract and induce the differentiation of odontoblasts[45]. Chemical disinfection of the root canal space may destroy these embedded growth factors.

REVASCULARIZATION AND ANGIOGENESIS

The concept of "revascularization" describes the clinical healing of periapical abscesses and continued root formation in immature teeth with nonvital pulps[91]. However, it does not encompass the actual healing and repair process that takes place in these clinical cases[92].

The revascularization method assumes that the root canal space has been disinfected and the formation of blood clot can produce a matrix (*e.g.*, fibrin) that traps cells capable of initiating new tissue formation. Its treatment effect is different from apexification because not only is the apex closed but the canal walls are thicker as well. It is also different from apexogenesis which also comes up with a closed apex and thicker dentinal walls resulting from the function of remaining vital root pulp.

With regard to revascularization, all the studies report the continued thickening of the dentinal walls and subsequent apical closure. The root length is increased by the growth of cementum. Connective tissue similar to PDL is also present in the canal space[93].

The success of root canal revascularization is mainly due to several factors. First, the immature avulsed tooth has an open apex, short root and intact but necrotic pulp tissue, so that the new tissue has easy access to the root canal system and a relatively short distance for proliferation to reach the coronal pulp horn. The speed with which the tissue completely revascularizes the pulp space is important because bacteria from outside are continually attempting to enter the pulp space. The ischemically necrotic pulp acts as a scaffold into which the new tissue grows, and the usually intact crown slows bacterial penetration because the only access for bacteria to the pulp is through cracks or enamel defects. Thus, the race between proliferation of new tissue and infection of the pulp space favors the new tissue formation. Second, minimum instrumentation preserves the viable pulp tissue which contributes to further development of open apex root. Third, young patients have greater healing capacity and more stem cell regenerative potential[94].

The greatest benefit of such biological approaches for dental tissue restoration over many conventional dental materials lies in the fact that reparative matrices become an integral part of the tooth, avoiding any of the problems arising from restoration retention and possible marginal bacterial microleakage. Moreover, this treatment approach strengthens the root walls of immature teeth.

NEURANAGENESIS

Pulp regeneration is not only to solve the aesthetic issues of the conventional root canal filling materials, but also to achieve the regeneration of the whole tooth vitality and restore the normal function of teeth. Dental pulp nerve regeneration can produce a protective response to maintain long-term survival of teeth when they were stimulated by mechanical, temperature, or chemical stimuli.

CLINICAL MANAGEMENT

Apexogenesis should be performed in three kinds of dental diseases of immature teeth, including reversible pulpitis, irreversible pulpitis and apical periodontitis. Pulp capping is usually applied for treating reversible pulpitis. The treatment of exposed vital pulp is accomplished by sealing the pulpal wound with CH or MTA to facilitate the reparative dentin formation. Irreversible pulpitis is often cured by pulpotomy following the steps below: (1) cervical pulpotomy to remove diseased pulp; 2) root canal disinfection with sodium hypochlorite; (3) placement of a thin layer of MTA in the crown aspect of the canal with a moist cotton pellet for 1 wk; (4) removal of the cotton pellet and sealing the root canal access with resin-modified glass ionomer; and (5) restoring the tooth with composite resin.

Traditional multiple-visit apexification with CH is the treatment choice of immature teeth suffering from periapical periodontitis, which can induce the formation of an apical hard tissue barrier. Due to the disadvantages listed above, regeneration management is recommended. Here are the protocols: (1) disinfect the root canal with sodium hypochlorite; (2) apply antibiotic paste (ciprofloxacin, metronidazole and minocycline) for 4 wk; (3) stir a file beyond the tooth apex to cause bleeding in the canal; (4) place a thin layer of MTA in the crown aspect of the canal; (5) seal the root canal access with resin-modified glass ionomer; and (6) restore the tooth with composite resin[33,95-97]. It is recommended that pulp regeneration should not be delivered to deciduous teeth as it may risk the retaining teeth and impair the eruption pattern of adult teeth[38,98].

PERSPECTIVES

Nowadays, various clinical studies are conducted using MSCs as transplants for treatment or to improve the functional outcomes. Stem cell-based therapies have drawn more attention to healing dental diseases. With the application of effective dental materials, stem cell-based apexogenesis may help a number of immature teeth develop. However, further work is required to increase the success rate of apexogenesis, so that this method can be widely used in the clinic.

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ORIGINAL ARTICLE

Choosing inclusion criteria that minimize the time and cost of clinical trials

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Abstract

AIM: To present statistical tools to model and optimize the cost of a randomized clinical trial as a function of the stringency of patient inclusion criteria.

METHODS: We consider a two treatment, dichotomous outcome trial that includes a proportion of patients who are strong responders to the tested intervention. Patients are screened for inclusion using an arbitrary number of test results that are combined into an aggregate suitability score. The screening score is regarded as a diagnostic test for the responsive phenotype, having a specific cutoff value for inclusion and a particular sensitivity and specificity. The cutoff is a measure of stringency of inclusion criteria. Total cost is modeled as a function of the cutoff value, number of patients screened, the number of patients included, the case occurrence rate, response probabilities for control and experimental treatments, and the trial duration required to produce a statistically significant result with a specified power. Regression methods are developed to estimate relevant model parameters from pilot data in an adaptive trial design.

RESULTS: The patient numbers and total cost are strongly related to the choice of the cutoff for inclusion. Clear cost minimums exist between 5.6 and 6.1 on a representative 10-point scale of exclusiveness. Potential cost savings for typical trial scenarios range in millions of dollars. As the response rate for controls approaches 50%, the proper choice of inclusion criteria can mean the difference between a successful trial and a failed trial.

CONCLUSION: Early formal estimation of optimal inclusion criteria allows planning of clinical trials to avoid high costs, excessive delays, and moral hazards of Type II errors.

Key words: Adaptive trial designs; Biomarkers; Clinical trials; Device; Drug therapy; Ethics; Methodology; Optimal allocation; Personalized medicine; Sequential design

Core tip: This paper presents statistical tools to model and optimize the cost of a randomized clinical trial as a function of the stringency of patient inclusion criteria. The patient numbers and total cost are strongly related to the choice of the cutoff for inclusion. Clear cost minimums exist for many realistic scenarios. Potential cost savings for typical trial scenarios range in millions of dollars. Early formal estimation of optimal inclusion criteria allows planning of clinical trials to avoid high costs, excessive delays, and moral hazards of type Ⅱ errors.

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INTRODUCTION

Clinical trials are too costly and take too long to com-

plete. High costs of clinical trials add significantly to the ultimate costs of new medicines and medical devices. Delay in completion of a trial due to inefficient trial design can postpone, sometimes indefinitely, the transfer of promising new therapies from bench to bedside. Assuming that a true positive treatment effect exists, strategies are needed for finding the most direct route to a statistically significant result using the smallest numbers of patients.

When a genuinely responsive subset of patients is diluted with many patients who are genetically or physiologically ill suited to respond to a new experimental treatment, the numbers of patients that must be studied to disprove the null hypothesis increases dramatically. Type Ⅱ errors in statistical inference (accepting the null hypothesis when it is false) can arise, and a useful drug, device, or procedure, which could have benefited some classes of patients, may be lost to further development. This situation is especially likely when only a fraction of patients in the treatment group respond well to the tested intervention, and when the control or comparison group is treated with a known, effective standard therapy, as is often done for ethical reasons. In this situation patient selection criteria are crucial.

An era of personalized medicine is emerging in which novel biochemical markers will be found for the diagnosis of cancer and other diseases[1]. When a genetic variation is linked to a specific drug effect, it becomes a biomarker that helps predict how an individual will react to a drug[2]. The treatment of cancer, in particular, is moving towards the use of more specific therapies that are targeted to each tumor type. To facilitate this shift, tests are being developed to identify those individuals who are most likely to benefit from particular treatments on the basis of the genes expressed by their tumors[3]. Such biomarkers may identify patients who will experience the most drug benefit and fewest side effects. In this setting innovative thinking about clinical trial design is needed to increase the proportion of patients receiving the best individual treatment, and to complete the trial more rapidly with fewer patients. There is also an ethical dimension to more efficient trial design: increasing the probability of a patient's being allocated to a successful treatment. With targeted, personalized therapy the study patients do not have to pay a high price for the benefit of future patients[4]. The challenge moving forward is to identify optimal trial design in a population with known biomarker levels, based upon screening data, and to identify the optimal allocation of patients to treatment groups, based upon mathematical and computer simulation of the trial.

Here we consider a paradigm in which either a phase Ⅱ trial data or an adaptive trial design provides pilot data describing responsiveness to the tested intervention in various types of patients. We consider the planning of a follow-on phase Ⅲ trial, in particular a two-treatment randomized clinical trial, including a control group and an experimental group and having a dichotomous end point such as response *vs* non-response to treatment. The definition of response is at the discretion of the investigator and is based on clinically desirable outcomes. Examples include disease free survival from cancer for a period of one year, induction of a state of clinical remission in leukemia, or resuscitation from cardiac arrest with a measurable pulse and blood pressure. The primary endpoints of the future trial are the proportions of patients that respond in the experimental group and in the control group.

The goal of the present research is to create a formal mathematical model of the planned randomized trial that will allow one to define and predict an optimal set of inclusion criteria. Such criteria would screen out non-responsive patient types and achieve a statistically significant result with the smallest number of patients and the lowest overall cost in both time and resources. In such a trial patients who are prospective candidates having an appropriate diagnosis would be screened according a list of possible metrics, such as age, tumor stage, or biomarker level. The screening metrics, here denoted x_1, x_2, …, *etc.* are combined mathematically by a classifier function, $F(x_1, x_2, \ldots)$, based on pilot data to obtain a single overall score, $x = F(x_1, x_2, \ldots)$, which is a predictor of successful response. Future patients for whom x equals or exceeds a cutoff value x_c will be included in the trial, and patients for whom $x < x_c$ will be excluded. Combinations of x_1, x_2, …, *etc.* yielding values of $x \geqslant x_c$ constitute the inclusion criteria for the study. The questions addressed by this paper are how to define a satisfactory classifier $F(x_1, x_2, \ldots)$ and how to best choose x_c to produce a statistically significant positive result with minimal time and cost, assuming an alternative hypothesis of a true treatment effect.

To help predict the most favorable inclusion criteria, it is helpful to regard the screening process and the function $F(x_1, x_2, \ldots)$ as a diagnostic test, for which the concepts of sensitivity and specificity apply. This paper demonstrates how one can use data from a one-armed phase Ⅱ study or early-stage pilot data from an adaptive trail design to create a suitable classifier $F(x_1, x_2, \ldots)$ for discriminating responders from non-responders and also to predict the best cutoff, x_c, for inclusion of future patients.

MATERIALS AND METHODS

Formulation of the problem

Suppose that a planned, two-arm, randomized clinical trial begins with evaluation of N possible candidates having a standard clinical diagnosis such as biopsy proven carcinoma of the breast. Suppose further that this population is heterogeneous in the sense that a proportion, q, of the patients are biologically well suited to respond to the experimental treatment (call them type 1 patients) having success probability $\pi_1 \approx 1$, and the remaining proportion, 1 - q, of the patients are biologically ill suited to respond to the experimental treatment (call them type 2 patients) having success probability $\pi_2 \approx 0$. It is normally not possible to predict in advance which patients will respond, but one can try to establish favorable inclusion criteria based on certain screening data. These data may

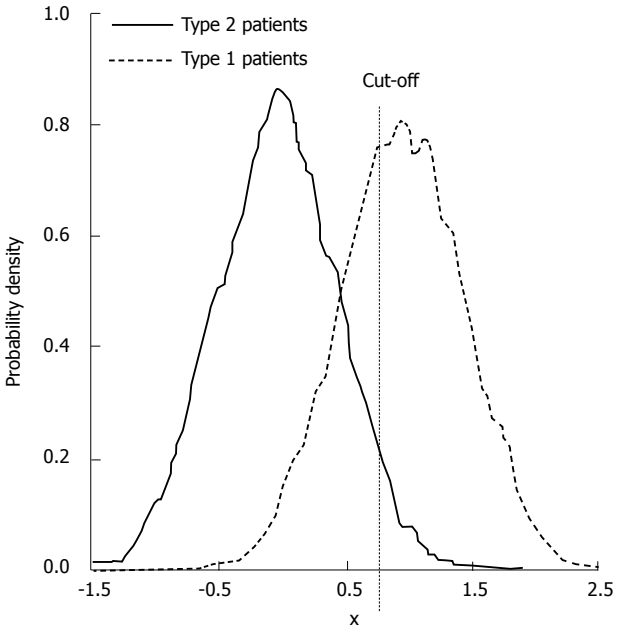

Figure 1 Separation of patient response phenotypes to a tested treatment according to an aggregate predictive variable, x. The fraction of type 1 responders to the right of the cutoff is the true positive fraction. The fraction of type 2 non-responders to the right of the cutoff is the false positive fraction. In this general example the units of x are arbitrary.

be a simple as age, gender, and stage of disease or may include sophisticated measures of biomarkers.

If the screening procedure had 100% sensitivity and 100% specificity for detecting guaranteed responsive type 1 individuals, who are very likely to respond to the new therapy, then the inclusion decision would be trivial: only type 1s would be included. In the more common situation potential good responders are difficult to identify, and a battery of imperfect metrics is employed. Suppose that such a battery of tests exists and that the test results x_1, x_2, *etc.* are combined in a single overall suitability test score, $x = F(x_1, x_2, \ldots)$. Type 1 and type 2 patients are likely to be distributed along the x-scale as shown in Figure 1, with significant overlap. Overlap of the distributions leads to meaningful fractions of false positive evaluations and false negative evaluations for the presence of the treatment responsive phenotype, given any chosen cutoff, x_c , for entry into the study.

In this sense we can regard the process of patient selection as a "diagnostic test", for which the concepts of sensitivity (true positive fraction, f_{tp}) and specificity (true negative fraction, f_{tn}) apply. The false positive fraction, $f_{fp} = 1 - f_{tn}$. If q is the fraction of type 1 individuals in the initial population of N patients and if 1 - q is the fraction of type 2 individuals, then Nqf_{tp} type 1s and $N(1 - q)f_{fp}$ type 2s will be selected for inclusion in the trial. As the cutoff x_c, is raised, the entry criteria become more strict, specificity for the responsive type 1 phenotype increases, but sensitivity decreases. Some potential good responders are excluded, and the overall study size is decreased, reducing its statistical power. In the limiting case overstrict inclusion criteria will reject nearly all patients. The time required to find perfect candidates will be excessive, and study numbers will be small. On the other hand, as the cutoff x_c, is reduced, the entry criteria become more loose. Sensitivity increases, but specificity decreases. The population of patients included in the trail is diluted with more and more non-responding type 2 patients. If q is small, the time and cost required to establish a significant treatment effect may become prohibitive.

It is reasonable to use N, the number of candidates initially considered for the trial before the screening process, as a measure of the cost of screening and also as one measure of the time required to complete the study. (If extensive long term follow-up is required, a constant plus N can be substituted.) It is also reasonable to use $N' = Nqf_{tp} + N(1 - q)f_{fp}$, the actual number of patients enrolled in the study, as a measure of the cost of treating and managing the patients over the course of the trial.

The mathematical treatment that follows includes several parts with the following objectives: (1) to create a formal mathematical model of the proposed randomized trial, given preliminary screening and outcome data; (2) to illustrate how such a model can be used to estimate the probability distribution of a test statistic describing the outcome of the trial; (3) to exercise the model to predict the number, N, of patients that must be screened and the number, N', of patients that must be included to reject the null hypothesis with a specified power, given the sensitivity and specificity of the screening process; (4) to characterize the sensitivity and specificity of the screening process as a receiver operating characteristic (ROC) curve; and (5) to compute the cost of the trial as a function of N and N' and to demonstrate how the cost varies as a function of the stringency of the inclusion criteria, based on the cutoff x_c, and in turn to determine if there is a "best" cutoff, x_c, for which a cost function of N and N' is minimized.

Creating a model using binomial distributions

Suppose, as before, that N patients are available to be screened for inclusion in a future randomized clinical trial comparing experimental and control groups. The end point of the trial is dichotomous. A fraction, $0 < q < 1$, of patients will respond well to the experimental treatment based on their genetics or physiology. Denote these good responding individuals as type 1 patients and remainder of non-responding individuals as type 2 patients. A screening procedure is performed having overall sensitivity f_{tp}, specificity f_{tn}, and false positive fraction $f_{fp} = 1 - f_{tn}$. After screening and evaluation $n = f_{tp}Nq$ type 1 patients and $m = f_{fp}N(1 - q)$ type 2 patients will be selected for inclusion in the trial. These selected patients will be randomized into control and treatment groups, which for generality need not be equal, having $\alpha(n + m)$ patients in the experimental group and $(1 - \alpha)(n + m)$ patients in the control group for $0 < \alpha < 1$.

Consider a model in which the probability of favorable outcome after the experimental treatment among type 1s is $\pi_1 = 1$, and the probability of favorable outcome after the experimental treatment among type 2s is $\pi_2 = 0$. To allow for the possibility that the type 1s and

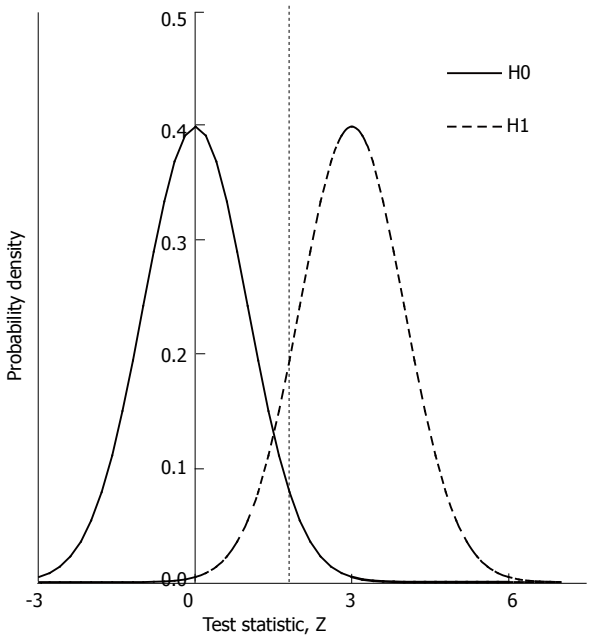

Figure 2 Calculation of power from probability density distributions for the null hypothesis (H0) and for an alternative hypothesis (H1). The dashed line shows critical value for significance (1.96 for two-tailed $P < 0.05$). The area under the thick curve to the right of the critical value is the statistical power of the test of H0.

type 2s may also respond differently after the control treatment, let the probability of favorable outcome after the control treatment among type 1s be π_3 and the probability of favorable outcome to the control treatment among type 2s be π_4 (Numerical values for π_3 and π_4 will be estimated from pilot data or published literature as described later). The expected outcome of the trial is shown in Table 1, showing the mean number of observed responders (successful outcomes) in each group.

Predicting statistical outcomes of the trial

Let us use the difference in proportion test for statistical inference for the purpose of predicting trial size and cost. (This choice in no way prevents the use of other statistical measures and tests of significance for reporting later results, including internal meta-analysis of the various stages[5,6]). The difference in the proportion of responders $\Delta p = p_E - p_C$ between experimental and control groups is computed and then divided by an estimate, $\hat{\sigma}$, of the standard deviation, σ, of the difference of proportions to obtain a test statistic $z = \Delta p/\hat{\sigma}$. Under the null hypothesis, H_0, the expected value of the z-statistic is zero and the standard deviation of the z-statistic is 1, as shown by the thin curve in Figure 2.

To explore the predicted N required for a statistically significant study as a function of model parameters, we can compute the distribution of the test statistic, z, under the alternative hypothesis, H_1, of a positive effect of experimental treatment. The form of this distribution, represented by the thick curve in Figure 2, is a function of model parameters, including probabilities π_1, π_2, π_3, and π_4, the number, N, of patients screened and the cutoff

Table 1 Expected values of enumeration data in a model trial

	Experimental group	Control group
Number of successes (responses)	$\alpha(n\pi_1 + m\pi_2)$	$(1-\alpha)(n\pi_3 + m\pi_4)$
Total	$\alpha(n + m)$	$(1-\alpha)(n + m)$

for patient inclusion. The distribution of z is characterized by its mean and variance, as follows.

From Table 1 the expected value, μ, of the difference in sampled proportions between the experimental and control groups is

$$\mu = [n(\pi_1 - \pi_3) + m(\pi_2 - \pi_4)]/(n + m) \quad (1a).$$

The variance of the difference in proportions is the sum of the variances of the independent sample proportions $\sigma^2 = \sigma^2(p_E) + \sigma^2(p_C)$. To find the variances note that the true population probabilities for responses in the experimental group and the control group are

$$\pi_E = (n\pi_1 + m\pi_2)/(m + n)$$

and

$$\pi_C = (n\pi_3 + m\pi_4)/(m + n) \quad (1b).$$

Hence, using the standard formulas for the variances of binomial distributions[7],

$$\sigma^2(p_E) = [\pi_E(1 - \pi_E)]/[\alpha(n + m)]$$

and

$$\sigma^2(p_C) = [\pi_C(1 - \pi_C)]/[(1 - \alpha)(n + m)] \quad (1c)$$

with

$$1-\pi_E = (n + m - n\pi_1 - m\pi_2)/(n + m)$$
$$= [n(1 - \pi_1) + m(1 - \pi_2)]/(n + m)$$

and similarly for $1 - \pi_C$.

Under the null hypothesis of zero treatment effect compared to control, the expected value of $p_E - p_C = 0$, and the test statistic

$$z = \frac{p_E - p_C}{\sqrt{\sigma^2(p_E) + \sigma^2(p_C)}} \quad (2)$$

will have mean value $\bar{z}_0 = 0$ and a standard deviation of one. That is, z will be distributed to good approximation as the standard normal distribution under H_0.

Under the alternative hypothesis of an expected positive treatment effect the expected value, μ, of $p_E - p_C$ will be greater than zero, and the test statistic, z, will have mean value, $\bar{z}_1 > 0$. The value of z_1 under H_1 is related to the values of parameters (1a) through (1c) and and to the critical values for significance testing and the statistical power of the trial. For example, for $P < 0.05$ the critical value is 1.96, and for a power of 84%, that is an

84% probability of detecting a true effect as significant, then $\bar{z}_1$ must be 1.0 standard deviation to the right of the cutoff in Figure 2, so that $\bar{z}_1 = 2.96$. To find the N and inclusion cutoff required to identify as statistically significant a particular treatment effect with a particular power we can explicitly evaluate $\bar{z}_1$ in terms of model parameters. Then

$$\bar{z}_1 = \frac{\mu}{\sigma} = \frac{\left[\frac{n(\pi_1 - \pi_3) + m(\pi_2 - \pi_4)}{m + n}\right]}{\sqrt{\frac{\pi_E(1 - \pi_E)}{\alpha(n + m)} + \frac{\pi_C(1 - \pi_C)}{(1 - \alpha)(n + m)}}} \quad (3a)$$

Knowing the target location of $\bar{z}_1$, one can estimate the statistical distribution of the results of the proposed trial, based upon the model parameters and the pilot screening and outcome data.

Predicting N and N' required to reject the null hypothesis with a specified power

After squaring (3a), substituting expressions (1), and simplifying the algebra,

$$\bar{z}_1^2 = \frac{[n(\pi_1 - \pi_3) + (\pi_2 - \pi_4)]^2}{n^2\left[\frac{\pi_1(1 - \pi_1)}{\alpha} + \frac{\pi_3(1 - \pi_3)}{1 - \alpha}\right] + nm\left[\frac{\pi_1 + \pi_2}{\alpha} + \frac{\pi_3 + \pi_4}{1 - \alpha} - 2\frac{\pi_1\pi_2}{\alpha} - 2\frac{\pi_3\pi_4}{1 - \alpha}\right] + m^2\left[\frac{\pi_2(1 - \pi_2)}{\alpha} + \frac{\pi_4(1 - \pi_4)}{1 - \alpha}\right]} \quad (3b)$$

Then substituting $n = f_{tp}qN$ and $m = f_{fp}N(1 - q)$ gives,

$$\bar{z}_1^2 = N \cdot \frac{[f_{tp}q + f_{fp}(1-q)] \cdot [f_{tp}q(\pi_1 - \pi_3) + f_{fp}(1 - q)(\pi_2 - \pi_4)]^2}{f_{tp}^2q^2\left[\frac{\pi_1(1-\pi_1)}{\alpha} + \frac{\pi_3(1-\pi_3)}{1 - \alpha}\right] + f_{tp}f_{fp}q(1 - q)\left[\frac{\pi_1 + \pi_2}{\alpha} + \frac{\pi_3 + \pi_4}{1 - \alpha} - 2\frac{\pi_1\pi_2}{\alpha} - 2\frac{\pi_3\pi_4}{1 - \alpha}\right] + f_{fp}^2(1 - q)^2\left[\frac{\pi_2(1-\pi_2)}{\alpha} + \frac{\pi_4(1-\pi_4)}{1 - \alpha}\right]} \quad (4)$$

which can be solved for N as a function of model parameters f_{tp}, f_{fp}, q, z_c, π_1 through π_4 , and the target power and level of significance represented by $\bar{z}_1$.

Expression (4) predicts N as a function of the proportion, q, of good responders in the population, the sensitivity and specificity of the screening procedure for inclusion into the study, and the effectiveness of the treatment in controls. Note since we use the square of $\bar{z}_1$ to get N, the resulting N could be that for a significant positive result with $p_E > p_C$ or a significant negative result with $p_C > p_E$. As expected, the required N becomes infinite, given the other parameters, when the null hypothesis is exactly true and the expected value of p_E equals the expected value of p_C.

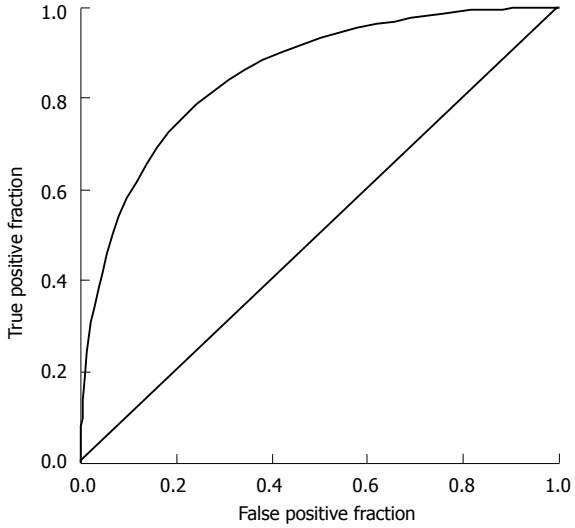

Figure 3 A sample receiver operating characteristic curve for a hypothetical screening test. In this example type 1 patients had screening scores, x, with a mean of 5.5 and a standard deviation of 1; type 2 patients had screening scores, x, with a mean of 4 and a standard deviation of 1. As the cutoff value xc is swept from 1.0 toward zero, a family of true positive and false positive fractions is created to generate the receiver operating characteristic (ROC) curve.

Characterizing the screening process as an ROC curve

To explore the effects of more selective *vs* less selective inclusion criteria, one can examine paired combinations of true positive fractions and false positive fractions for a typical screening procedure as defined by a ROC curve. An ROC curve is a plot of f_{tp} as a function of f_{fp} in the unit square, as the cutoff value of decision variable, x, is gradually reduced from the maximum possible value of x toward the minimum possible value. A typical ROC curve is illustrated in Figure 3. Each point on the curve represents a realistic combination of f_{tp} and f_{fp} (sensitivity and 1- specificity) for a particular classifier used to distinguish type 1 *vs* type 2 patients.

In this context the ROC curve describes a family of cutoff values in the x-domain for partially overlapping distributions of good responding, type 1 patients and non-responding, type 2 patients. An ROC curve that is shifted upward and to the left indicates a better discriminating screening test. The ROC curve provides a useful mathematical model of stricter *vs* looser inclusion criteria for a clinical trial.

With this model one can explore the influence of inclusion criteria on the size and cost of the clinical trial. The top curve in Figure 4 is a representative plot of N from expression (4) as a function of cutoff value x_c. N represents the number of patients screened in a hypothetical clinical trial with a statistically significant positive result ($P = 0.05$).

The number of patients actually enrolled in the trial after screening, according to the definitions of the model, is

$$N' = n + m = f_{tp}Nq + f_{fp}N(1 - q) \quad (5).$$

This number is plotted as the bottom curve in Figure

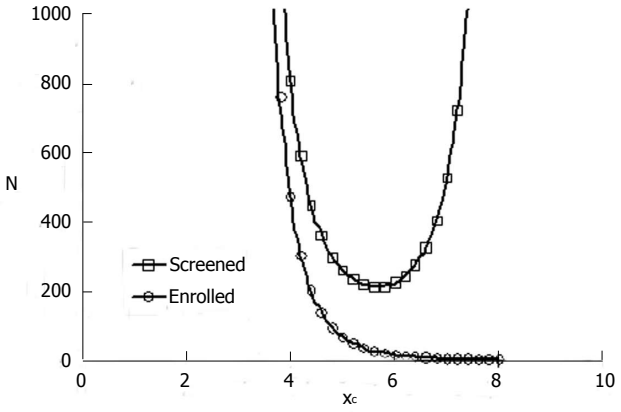

Figure 4 Numbers of patients screened and enrolled in a model study of heterogeneous responders having a statistically significant positive result. For this model the proportion of type 1, good responders q = 0.2, the response probability for type 1 patients, π_1 = 1.0, the response probability for type 2, poor responders, π_2 = 0. The response probabilities for both phenotypes to the control treatment, π_3 and π_4 both equal 0.2. The mean value of the z statistic for the alternative hypothesis is 2.96 (84% power for the trial). The proportion of patients, α, assigned to the experimental group is 0.5.

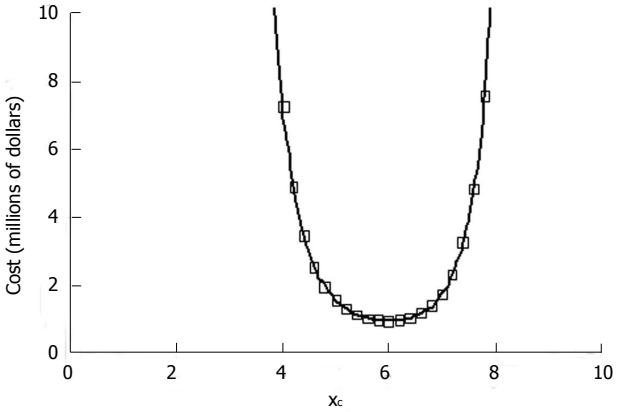

Figure 5 Cost estimates in a model study of heterogeneous responders. Cost constants in thousands of dollars are as follows: screening cost per case c_1 = 1, treatment cost c_2 = 10, opportunity cost c_3 = 100/yr, case rate r = 50/yr, follow up time t =1 yr. Other details as in Figure 4.

4 for one hypothetical example.

In this example the mean composite screening score, x_c, for responders is 5.5 and the standard deviation is 1. The mean composite screening score for non-responders is 4.0 with a standard deviation of 1. The ROC curve for this scenario is that of Figure 3. A value of cutoff $x_c < 2$ means that all comers were included in the study. That is, there was no selection. A cutoff > 8 means that virtually all patients were excluded. In the mid range of inclusion criteria, there remains a strong effect of screening selectivity on the number of patients required to produce a significant result, given the alternative hypothesis. There is a clear optimal cutoff for patient selection near $x_c = 5.6$ that minimizes the number of patients, N, with an initial diagnosis needed to produce a statistically significant positive result.

Computing the cost of the trial

A total cost model is easily developed from the forgoing. The value of N as a function of f_{tp} and f_{fp} is a measure of the cost of screening, since all suitable patients must be screened. The value of N' as a function of f_{tp} and f_{fp} is a measure of the cost for treatment and monitoring of enrolled patients, since more enrolled patients will require more personnel, facilities, coordination, data management, *etc*. The opportunity cost of delayed revenue from a successful new product and the opportunity cost of diversion of resources from other worthwhile projects are related to the duration of the trial.

Let c_1 be the cost of screening per patient. Let c_2 be the average cost of treatment per patient in both control and experimental groups. Let c_3 be the opportunity cost per year in delay of marketing a successful drug or device, that is, the expected revenue divided by the duration of the study. Let r be the case rate, that is, the rate at which new cases appear for screening, and let t be the time required for follow up of a patient after entry into the study. In this case the total cost of the study is

$$\text{Cost} = c_1N + c_2N' + c_3(N/r + t). \quad (6)$$

Additional cost terms can be added, if desired, such as performance site start-up costs, which would be related to N divided by the number of proposed sites. Figure 5 shows for the preceding example in Figure 4 the total cost calculation for the hypothetical cost constants given in the figure legend.

The anticipated cost of the study is strongly dependent on the stringency of the inclusion criteria. A low cost sweet spot exists for a narrow range of inclusion cutoff values in the range of 5.6 to 6.1 for this model. The result is consistent with qualitative experience that good results occur in a reasonable amount of time when patient selection is targeted and rigorous, but not so rigorous as to choke off the number of patients entering the trial who might benefit.

A particularly interesting situation arises when the average response probability for all patients given the experimental treatment, which is equal to q, is less than that for type 2 patients given the control treatment. In Figure 6 we have the situation in which π_1 = 1.0 and π_2 = 0, and q = 0.2, as before. However, we have $\pi_3 = \pi_4 = 0.4$. The experimental drug is much less effective than control treatment for type 2 patients. The cost projections include a vertical asymptote when the null hypothesis is exactly true, that is the expected value of p_E equals the expected value of p_C. To the right of the dashed line a significant positive effect, $p_E > p_C$, can be detected at the indicated cost. To the left of the dashed line a significant negative effect, $p_C > p_E$, can be detected. In such situations, which may be quite common in practice, choice of inclusion criteria could well make the difference between a futile study and a successful one. Thus the choice of inclusion criteria clearly can have large effects on the cost and success of a clinical trial.

The next sections develop methods to construct a classifier $F(x_1, x_2, \ldots)$ and to estimate the model param-

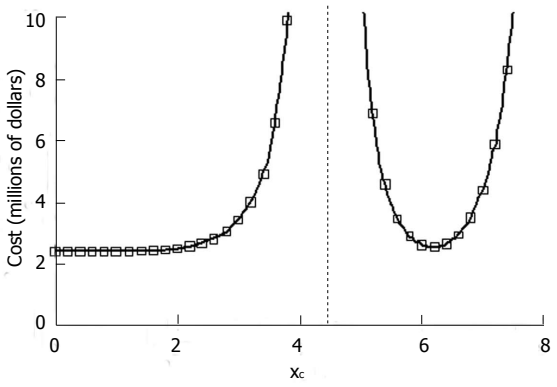

Figure 6 Cost estimates in a scenario with good responsiveness to the control treatment in patients who are non-responsive to the experimental treatment. $\pi_1 = 1.0$, $\pi_2 = 0$, $\pi_3 = \pi_4 = 0.4$. Other details as in Figure 5. Dashed line divides the x-domain into regions of a significant negative effect (to the left) *vs* a significant positive effect (right). Near $x_c = 4.4$ the cost of disproving the null hypothesis when it is exactly true becomes infinite.

eters in an adaptive clinical trial, based on a phase Ⅱ pilot data for treatment outcome and for screening variables, x_1, x_2, …*etc*. Such calculations would allow estimation of the optimal choice of inclusion criteria in a phase Ⅲ trial for lowest cost and highest efficiency.

Building a classifier using screening data

Model based prediction of optimal inclusion criteria requires the creation of an effective classifier to screen for type 1 patients based upon pilot data. Here we derive a relatively simple and effective linear classifier for combining an arbitrary number of screening variables, x_1, x_2, … x_k, to obtain a single overall predictor $x = F(x_1, x_2, \ldots x_k)$. Using the distributions of combined screening results, x, for responders and for non-responders to the experimental treatment, one can estimate the ROC curve for detection of good responding, type 1 patients. Here "responders" are those patients observed to have a successful outcome from the experimental treatment. "Non-responders" are those patients observed to have a poor outcome from the experimental treatment.

To create a classifier one must first examine screening data and outcomes in response to the experimental treatment in available preliminary data for all comers. The association between satisfactory response and possible predictors x_1, x_2, … , such as age, sex, tumor stage, or biomarker level, can be judged by plotting the distributions of each variable for responders and non-responders. Continuous variables are dichotomized in a convenient way, using the joint median or a cutoff suggested by the shapes of the screening data distributions, for example, age < 50 years = 0 (young) and age ⩾ 50 years = 1 (old). An apparent difference in the proportions of responders *vs* non-responders suggests that useful predictive information is captured by variable x_i. Combining three or four features, x_i , in different domains of anatomy and physiology will likely lead to more accurate prediction of response to therapy.

As shown in Appendix 1, a near optimal choice of a linear classifier function for k relatively independent or poorly correlated predictors, x_1 , x_2 …, x_k, is

$$x = F(x_1, x_2, \ldots x_k) = \sum_{i=1}^{k} a_i x_i . \quad (7)$$

where constant coefficients

$$a_i = \bar{x}_{iR} - \bar{x}_{iNR} = p_{iR} - p_{iNR},$$

and subscript R indicates responders to the experimental treatment in the preliminary data set and subscript NR indicates non-responders.

For dichotomous variables $\bar{x}_i \in (0,1)$ the mean value $\bar{X}_{iR}$ is the equal to the proportion, p_{iR}, of responders for whom $x_i = 1$, and mean value $\bar{x}_{iNR}$ is the equal to the proportion, p_{iNR}, of non-responders for whom $x_i = 1$. Each coefficient, a_i, is the observed difference between the average value of x_i for responders and the average value of x_i for non-responders. If two variables are highly correlated, for example blood urea nitrogen and serum creatinine concentration, they can be combined for simplicity and validity into a single dichotomous variable (renal insufficiency) with a reduction in k. In this way it is possible to construct an aggregate measure, x, that best separates the distribution of responders from that of non-responders. For k dichotomous screening measures there are 2^k possible values of x.

To avoid negative values, the variable x can be re-scaled to units of percent with 0 representing the minimum practical value of x and 100 representing the maximum practical value, based on coefficients a_i. Some of the a_i may be < 0. The maximal and minimal values of x must be determined by inspection. Then the re-scaled x-values $x\,(\%) = 100(x - x_{min})/(x_{max} - x_{min})$. Such units are helpful in any future clinical application of the x-scale, with a patient requiring a certain number of "points", x_c , on a 0 to 100 scale for inclusion in later stages of the trial.

In turn, one can estimate various possible combinations of false positive fraction, f_{fp}, and true positive fraction, f_{tp}, from the distributions of x-values for responders and for non-responders. Then the receiver operating characteristic (ROC) curve describing possible pairs of f_{tp} and f_{fp} from phase Ⅱ data can be constructed, using alternative cutoff values ranging from the maximum to the minimum observed values of x.

To obtain the true positive and false positive fractions, f_{tp} and f_{fp}, for any x_c one may proceed in particular as follows. If n_{NR} is the total number of non-responders to the experimental treatment in the pilot data set, n_R is the total number of responders to the experimental treatment in pilot data set, x_c is a chosen cutoff value in the x-domain, $n_{NR} \mid x \geqslant x_c$ is the number of non-responders for whom x equals or exceeds the cutoff value, and $n_R \mid x \geqslant x_c$ is the number of responders for whom x equals or exceeds the cutoff value, x_c, then

$$f_{fp}(xc) = \frac{n_B \mid x \geqslant xc}{n_B} \quad \text{and}$$

$$f_{fp}(xc) = \frac{n_A \mid x \geqslant xc}{n_A} \quad (8)$$

Estimating model parameters q, π_3, and π_4, from pilot data

Estimation of q: Recall that q is defined as the true proportion of good responding patients in the screened population. Using the complete pilot data set, the best estimate of q is the proportion of responders to the experimental treatment in the initial unscreened population for which preliminary data are available. This working estimate of q is denoted $\hat{q}$.

Estimation of π_3 and π_4: To obtain estimates for the remaining control group parameters π_3, and π_4, indicating the response probabilities for type 1 and type 2 patients to the control treatment, one needs to examine preliminary data, or else previously published data, for patients given the control treatment and for whom screening measures are known or can be estimated. For the patients in the control group, we can impose similar selection criteria based on cutoffs, x_c, and corresponding values of f_{tp}, f_{fp}, and $u = f_{fp}/f_p$, developed from the distributions of responders *vs* non-responders to experimental (not control) therapy. For the model of Table 1, where, as before, $n = f_{fp}qN$ and $m = f_{fp} N (1- q)$

$$Pc(u) = \frac{n\pi_3 + m\pi_4}{n + m} = \frac{f_{tp} Nq \pi_3 + f_{fp} N(1 - q)\pi_4}{f_{tp}Nq + f_{fp} N(1 - q)} = \frac{q\pi_3 + (1-q)\pi_4 u}{q + (1 - q)u} \quad (9)$$

If we define $\theta = \hat{q}/(1-\hat{q})$ for the working estimate, $\hat{q}$, then we can obtain working estimates, $\hat{\pi}_3$ and $\hat{\pi}_4$, from the observed relationship

$$P_C(u) = \frac{\hat{\pi}_3\theta + \hat{\pi}_4 u}{\theta + u} \quad (10)$$

or

$$y(u) - P_C(u).(\theta + u) - \hat{\pi}_3\theta - \hat{\pi}_4 u \quad . \quad (11)$$

Expression (11) implies that the following regression analysis may be used to estimate π_3 and π_4 from pilot data, given pairs of data points f_{tp} and f_{fp} , and in turn the ratio, u. Since θ is known from experimental group data, we can plot for control group data the product $y(u) = p_C(u).(\theta + u)$ as a function of u and fit a linear, least-squares line to the data. From the slope and intercept of the regression line we can obtain estimates, based on all the control data for

$$\hat{\pi}_3 = \text{intercept}/\theta \text{ and } \hat{\pi}_4 = \text{slope} \quad . \quad (12)$$

Often values $\hat{\pi}_3$ and $\hat{\pi}_4$ from (12) will differ because stronger patients respond better to both experimental and control drugs.

In this way one can obtain estimates of all model parameters based on preliminary or published data. For each pair of values, f_{tp} and f_{fp}, on the ROC curve corresponding to a given cutoff value x_c, one can evaluate expression (4) to obtain projected numbers N of patients that must be screened and using expression (5) the projected numbers N' of patients admitted to the trial that will be required to establish a statistically significant effect under the alternative hypothesis, H_1. Incorporation of the cost model (6) allows reasonable projections of future trial costs as a function of inclusion criteria, based upon available data. One then can continue in the future, operating under inclusion criteria determined by x_c. An adaptive phase Ⅲ trial design is possible in which the cutoff, x_c, is revised on the basis of accrued data at a later time.

RESULTS

Classification of pilot data

To demonstrate the technique and benefits of model based selection of inclusion criteria we can use a realistic data set that is similar, but not identical to that published by Shaw *et al*[8] Table 2 shows reconstructed raw data for this study of a novel drug for the treatment for lung cancer. Patients are characterized by age, sex, smoking history, and the presence of a specific cell surface receptor. These four predictor variables are dichotomized. The 16 possible combinations of predictors form 16 classes of patients indicated by the rows of Table 2. The class number is indicated in the left most column. The next four columns indicate values of the four dichotomous variables. Values of 1 denote old, male, smoking, or receptor (biomarker) positive patients. Values of 0 denote young, female, non-smoking, or receptor negative patients. The next two columns are the counts of patients treated with the experimental drug in each of the 16 possible classes. These were reconstructed from published summary data. The column labeled "NR count" indicates the numbers of non-responders in each class. The column labeled "R count" indicates the numbers of responders in each class. The next two columns are raw counts of patients in each class treated with the control chemotherapy regimen. Controls are similarly divided into non-responders (NR) and responders (R).

To create a classifier for predicting responders to the experimental drug from the dichotomous screening variables, the mean values of each dichotomous variable, age, sex, *etc.* for non-responders and responders to the experimental treatment are tabulated at the bottom of Table 2 in columns 2 through 5. These averages are equal to the proportions of patients labeled successes or failures with predictor variables of each column equal to 1. The responder minus non-responder differences in these variables are the coefficients a_1, a_2, a_3, and a_4 in the linear combination $x = a_1x_1 + a_2x_2 + a_3x_3 + a_4x_4$ (expression (7). The values of x for each class are computed using this function for each of the 16 classes of patients and shown in the second from the right hand column of Table 2. The rightmost column of Table 2 shows these x-values expressed in convenient units of percent, $100(x - x_{min})/(x_{max} - x_{min})$.

Table 2 Raw data from a reconstructed study of cancer treatment

Class	Old	Male	Smoke	Receptor	Exp NR count	Exp R count	Control NR count	Control R count	x	x%
1	1	1	1	1	0	0	0	0	-0.072	47
2	1	1	1	0	5	1	4	2	-0.619	0
3	1	1	0	1	0	0	0	0	0.211	71
4	1	1	0	0	6	1	4	2	-0.336	24
5	1	0	1	1	0	0	1	0	0.113	63
6	1	0	1	0	9	1	6	3	-0.434	16
7	1	0	0	1	1	3	2	2	0.396	87
8	1	0	0	0	9	1	7	3	-0.151	40
9	0	1	1	1	0	0	0	0	0.078	60
10	0	1	1	0	4	0	3	1	-0.468	13
11	0	1	0	1	0	1	1	0	0.362	84
12	0	1	0	0	4	0	3	2	-0.185	37
13	0	0	1	1	0	0	0	0	0.263	76
14	0	0	1	0	6	1	5	2	-0.283	29
15	0	0	0	1	3	6	4	4	0.547	100
16	0	0	0	0	4	1	5	3	0	53
pNR	0.588	0.373	0.471	0.078						
pR	0.438	0.188	0.188	0.625						
Coef-ficients	a_1 -0.151	a_2 -0.185	a_3 -0.283	a_4 0.547						

R: Responders; NR: Non-responder.

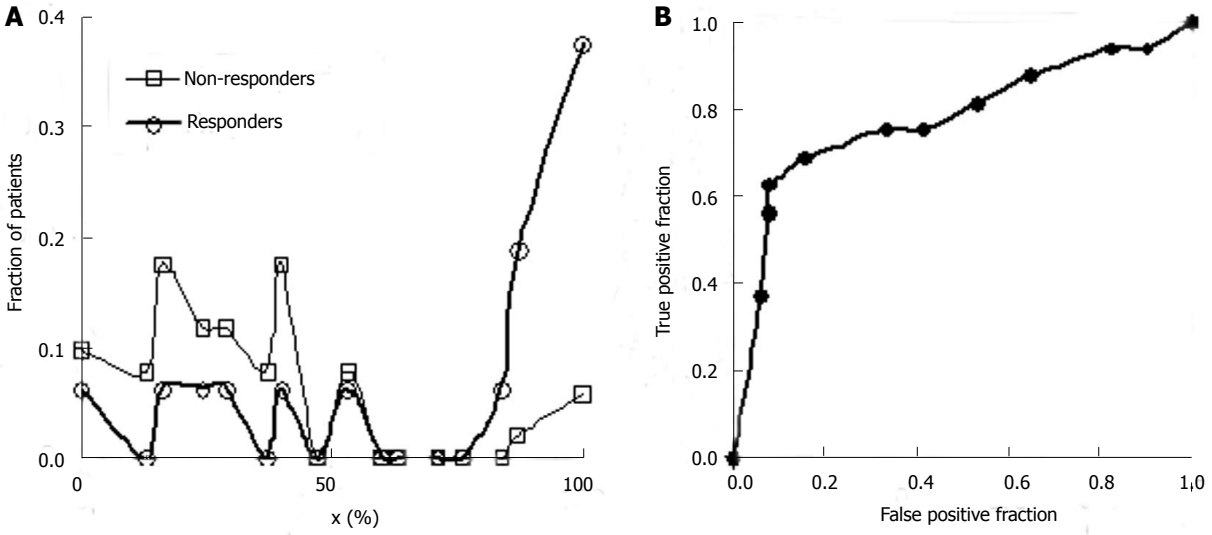

Figure 7 Fraction of patients. A: Separation of observed responders and non-responders to the experimental treatment along the x-domain in this reconstructed preliminary study. The fraction of patients with each x-value is show on the vertical axis. Patients with x-scores over 60% have a much greater likelihood of responding; B: ROC curve for the screening procedure.

The next step in the analysis requires sorting the classes by x-value from smallest to largest. Owing to the definition of the coefficients a_i, responders will be expected to cluster toward higher values of x and non-responders will be expected to cluster toward lower values of x. Table 3 shows sorted data for the experimental treatment group on the left and for the conventional (control) treatment group on the right. The rows are now sorted by x-values, determined from the experimental data in Table 2.

Computation of the ROC function

Columns 4 and 5 from the left in Table 3 give the fractions of non-responders and responders to the experimental treatment in each class. These values are equivalent to the probability density function defined over the set of classes. Figure 7A shows the separation of responders and non-responders to the experimental treatment along the x-domain. The fraction of patients with each x-value is shown on the vertical axis. Patients with x-scores less than 50% respond better to the control treatment. Patients with x-scores over 80 percent respond better to the experimental treatment. These results alone suggest that future studies of the experimental drug for lung cancer focus on patients with x-scores of 60 or better. Other patients are not likely to benefit, and if these are included in future trial statistics, a larger N will be re-

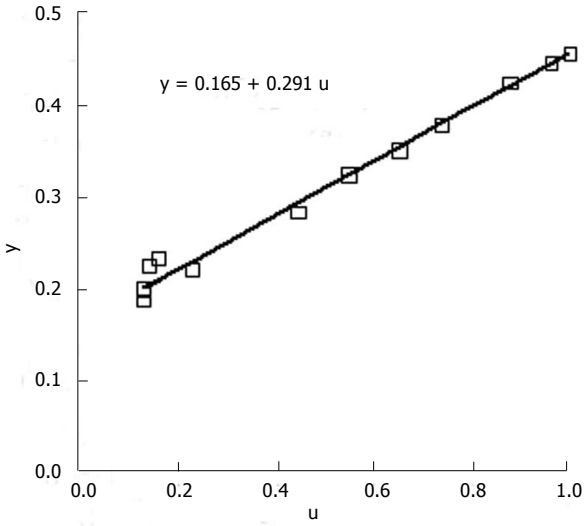

Figure 8 Regression analysis on the last two columns of Table 3. A plot of the hybrid variable, y = pC(u)(π + u), *vs* u can be used to evaluate model parameters π_3 and π_4. The slope of the regression line is π_4, and the intercept divided by π is π_3.

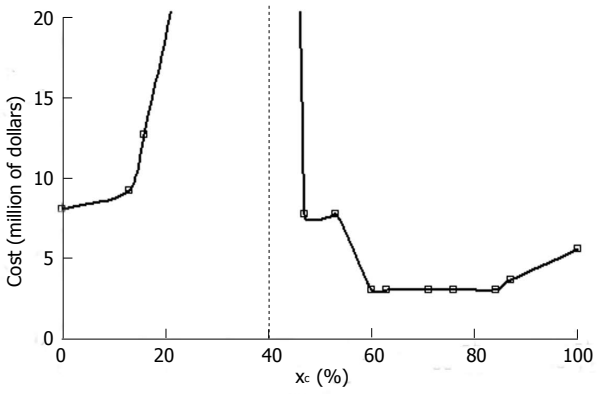

Figure 9 Cost estimates in a realistic test data set for targeted drug therapy of lung cancer, presented in Tables 2 and 3. Cost constants in thousands of dollars are as follows: screening cost per case c1 = 1, treatment cost c_2 = 10, opportunity cost c_3 = 100/year, case rate r = 50/year, follow up time t =1 year. Cost to the right of the dashed vertical asymptote are for a significant positive result (experimental treatment better than control). Costs to the left of the dashed vertical asymptote are for a significant negative result (experimental treatment worse than control).

quired to reject the null hypothesis at substantially greater time and cost.

By integrating the functions plotted in Figure 7A or constructing a running sum of values in Columns 4 and 5 of Table 3 one can obtain the true positive fractions and false positive fractions using expression (8) for patients for whom x equals or exceeds a cutoff value indicated in each row. The values of f_{fp} and f_{tp} are shown in the next two columns. From these values the ROC curve for screening (f_{tp} as a function of f_{fp}) can be plotted, as shown in Figure 7B. The values of f_{tp} and f_{fp} are needed to model the size and cost of the future clinical trial using equations (4), (5), and (6).

Estimation of q, the population proportion of responders

The value of parameter, q, is best estimated as the proportion of responders for all x-values, or the total of column 3 in Table 3 divided by the total of columns 2 and 3, namely $\hat{q} = 16/67 = 0.24$.

Regression analysis of control data for π_3 and π_4

The values of parameters π_3 and π_4 are obtained by the regression analysis of expressions (9) through (12), using the control treatment data on the right of Table 3. The values in column 9, labeled $p_C|x_c \geq x$, are the conditional probabilities of response given that the cutoff value of x is at least as great as the x in any particular row. These values are important to explore, because patients that are likely to respond to the experimental drug may also tend to respond to the control treatment, being stronger by virtue of qualities not measured by x_1 through x_4. These probabilities π_3 and π_4 of response to control treatment can be estimated from regression analysis of derived variables u and y. The value of u in column 10 equals f_{fp}/f_{tp}, based upon the true positive and false positive fractions from experimental (not control) data. The value y in column 11 of Table 3 is the hybrid variable $y = p_C(u)(\theta + u)$. The slope of the regression line of a plot of y *vs* u is an estimate of π_4, and the intercept divided by $\theta = q(1 - q)$ is an estimate of π_3.

Figure 8 shows the regression analysis on the rightmost two columns of Table 3. Both u and y are dimensionless. The intercept of the regression line is 0.165 and the slope is 0.291. Using expression (13), we have $\hat{\pi}_3 = \text{intercept}/\theta = 0.53$, and $\hat{\pi}_4 = \text{slope} = 0.29$. The lumped control proportion of responders for all comers is 0.35. As expected, those classified as strong responders to the experimental treatment are also somewhat more likely to respond to the control treatment, an effect that should be accounted in modeling.

Summary of model parameters

Parameters for the statistical model in this example are therefore q = 0.24, $\pi_1 = 1$, $\pi_2 = 0$, $\pi_3 = 0.53$, and $\pi_4 = 0.29$.

Exercising the model to predict cost

Figure 9 shows the corresponding cost function for model parameters q = 0.24, $\pi_1 = 1$, $\pi_2 = 0$, $\pi_3 = 0.53$, and $\pi_4 = 0.29$. Cost was computed using equations (4), (5), and (6) in succession. Cost coefficients are given in the figure legend.

This realistic example demonstrates that the choice of inclusion criteria can have a profound effect on the outcome of a clinical trial and that adjustment of inclusion criteria by quantitative means can produce protocols that achieve more with less. In Figure 9 the costs to the right of the dashed vertical asymptote correspond to a significant positive result with the experimental treatment better than control. Inclusion criteria of x > 60 points are likely to produce such outcomes. Costs to the left of the dashed vertical asymptote represent a significant negative result (experimental treatment worse than control).

Table 3 Analysis of data sorted by x-value

x (%)	Sorted experimental data and ROC curve						Sorted control data and regression analysis				
	NRcount	R count	p (NR|x)	p (R|x)	f_{fp}	f_{tp}	NR count	R count	pc|xc ⩾ x	u	y= pc (q + u)
0	5	1	0.098	0.063	1.000	1.000	4	2	0.348	1	0.457
13	4	0	0.078	0	0.902	0.938	3	1	0.349	0.962	0.446
16	9	1	0.176	0.063	0.824	0.938	6	3	0.356	0.878	0.424
24	6	1	0.118	0.063	0.647	0.875	4	2	0.360	0.739	0.379
29	6	1	0.118	0.063	0.529	0.813	5	2	0.364	0.652	0.351
37	4	0	0.078	0	0.412	0.750	3	2	0.378	0.549	0.326
40	9	1	0.176	0.063	0.333	0.750	7	3	0.375	0.444	0.284
47	0	0	0	0	0.157	0.688	0	0	0.409	0.228	0.222
53	4	1	0.078	0.063	0.157	0.688	5	3	0.409	0.228	0.222
60	0	0	0	0	0.078	0.625	0	0	0.429	0.125	0.188
63	0	0	0	0	0.078	0.625	1	0	0.429	0.125	0.188
71	0	0	0	0	0.078	0.625	0	0	0.462	0.125	0.203
76	0	0	0	0	0.078	0.625	0	0	0.462	0.125	0.203
84	0	1	0	0.063	0.078	0.625	1	0	0.462	0.125	0.203
87	1	3	0.02	0.188	0.078	0.563	2	2	0.500	0.139	0.227
100	3	6	0.059	0.375	0.059	0.375	4	4	0.500	0.157	0.235

R: Responders; NR: Non-responder.

Inclusion criteria of x < 20 points would likely produce a significant negative outcome. The dashed vertical line represents selection criteria that would produce results entirely consistent with the null hypothesis.

DISCUSSION

A major challenge to medical innovation in the modern era is that when new improved drugs or other treatments are compared with reasonable, effective standard therapy, larger and larger trials are needed to detect incremental benefits at skyrocketing costs. If the effect of experimental treatment is borderline overall and strong in one subgroup, the overall conclusion is that the experimental treatment is not significantly different from control. The potential benefit in the favored subgroup is often not pursued, owing to limitations of time and cost.

This dilemma has led to the development of adaptive trial designs[9-14]. If investigators can determine early-on which types of patients are most likely to benefit from a novel treatment, then the trail can be re-targeted to favorable patients only. Alternatively, if a particular phenotype, such as the diabetic state, is found to have untoward complications compared to other types, then such patients can be excluded going forward, on a rational basis.

Here we show using a model-based approach how it is possible to minimize the time, cost, and probability of type II error of a clinical trial, by selection of optimal patient inclusion criteria. This approach provides a route to planning of a staged clinical trial for efficient use of resources in the confirmation stage of an adaptive trial design. It might even provide a way to resurrect good drugs or devices from failed trials by re-analysis of inclusion criteria used in the past.

The present model based approach can also be applied to data from one-armed preliminary trials of efficacy. Patients receiving the experimental treatment are characterized according to potential measures x_1 through x_k for tightened inclusion criteria. The distributions of values x_i including all treated patients are tabulated and plotted for each metric, i. Continuous data such as age or fasting blood sugar concentration are dichotomized, based on inspection of the frequency distributions for responders *vs* non-responders to experimental treatment. Inherently dichotomous variables, such as male/female, or diabetic/non-diabetic are allowed also. Treated pilot patients are sorted into classes of putative responders and putative non-responders. Differences in proportions are used to construct a classifier (7), from which one can construct an ROC curve similar to Figure 3 using expression (8) that specifies possible pairs of f_{tp} and f_{fp} corresponding to different cutoffs for patient inclusion. These values, together with those of π_3, π_4 and q, estimated as described from pilot data and/or from the literature for standard (control) therapy, allow construction of the cost function (6) and identification of minimal cost inclusion criteria going forward.

The present work builds upon the rich literature describing adaptive clinical trial designs. An adaptive design allows the users to modify a trial during its progress based on interim results without affecting the validity and integrity of the trial. There are several subtypes of adaptive designs[15]. A group Sequential design allows for premature termination of a trial based on evidence of strong efficacy or futility at interim analyses. If a trial shows a positive result at an early stage, the trial is stopped, leading to an earlier launch of the new drug. If trail shows a negative result, early stopping avoids wasting resources. Sequential methods typically lead to savings in sample size, time, and cost when compared to the classical design with a fixed sample size[16].

Adaptive design with sample size re-estimation based upon interim results avoids inaccurate estimation of the effect size and its variability, which can lead to an underpowered or overpowered study. If a trial is underpowered, it will not be able to detect a clinically meaningful difference, and consequently could prevent a potentially effective drug from being delivered to patients. If a trial

is overpowered, it could lead to unnecessary exposure of many patients to a potentially harmful compound when the drug, in fact, is not effective. Adaptive sample size re-estimation avoids these pitfalls and can reduce the expected sample size, and in turn the cost of the study, under a range of treatment effects. Protocols and procedures for re-specification of sample size are well described in the literature[4,17-21]. This type of adaptive design can arguably reduce time and cost, but does not specifically deal with optimizing inclusion/exclusion criteria.

Other forms of adaptation deal with allocation of patients to particular treatment groups. A drop-the-loser design is an adaptive design consisting of multiple stages. At each stage, interim analyses are performed and the losers (*i.e.*, inferior treatment groups) are dropped. Note that this approach does not deal with patient selection but with treatment selection. Alternatively, a play-the-winner design increases allocation to successful treatments, based upon preliminary results. This form of adaptive design is most useful in multiple-arm or dose-ranging trials. They allow a shared control group, dropping of ineffective treatments before the end of the trial and stopping the trial early if sufficient evidence of a treatment being superior to control is found[22]. These now classical kinds of adaptive designs refine how many randomly selected patients are placed in known treatment groups. They do not refine patient selection criteria based upon biomarkers or traits that contain information about how individual patients are likely to respond to individual treatment.

Biomarker adaptive designs, currently being developed, allow adaptations according to biomarkers that indicate biologic or pharmacologic response to a therapeutic intervention. In one application biomarkers may serve as surrogate end points that predict outcomes such as long-term survival[23]. In another application, envisioned in the present study, biomarkers can be used to select the most appropriate target population. Recently, Jiang *et al*[24] proposed a statistically rigorous biomarker-adaptive threshold phase Ⅲ design, in which a putative biomarker is used to identify patients who are sensitive to the new agent. The biomarker is measured on a continuous or graded scale, and a cut point established to define the sensitive subpopulation. Using a proportional hazards model that describes the relationship among outcome, treatment, and biomarker value for a two-treatment clinical trial, they found that when the proportion of sensitive patients as identified by the biomarker is low, the proposed design provided a substantial improvement in efficiency compared with the traditional trial design. Drs. Freidlin *et al*[9] proposed a new adaptive design for randomized clinical trials of targeted agents in settings where an assay or signature that identifies sensitive patients is not available at the outset of the study. They concluded that when the proportion of patients sensitive to the new drug is low, the adaptive design substantially reduces the chance of false rejection of effective new treatments. This prior work, as well as the present study, supports the idea that biomarkers can add substantial value to current medical practice by guiding patient-specific treatment selection in the conduct of clinical trials[25].

As such biomarker adaptive trial designs become implemented, more patients will receive a treatment that is effective for them. Fewer useful therapies for carefully selected patients will be lost to further development. The transition from bench to bedside will be faster, future patients awaiting better treatments will have less time to wait, and the high cost of conducting clinical trials will be minimized.

Coefficients for an approximately optimal linear classifier

Let the linear classifier $x = \sum_{i=1}^{k} a_i x_i$ for dichotomous predictive variables $\bar{x}_i \in (0,1)$ and for x_i independent or poorly correlated, based upon pilot data. Treat the coefficients, a_i, as variables to be optimized for best discrimination of non-responders, NR, from responders, R. The mean values from pilot data for these subgroups are $\bar{x}_{NR} = \sum_{i=1}^{k} a_i \bar{x}_{iNR}$ and $\bar{x}_R = \sum_{i=1}^{k} a_i \bar{x}_{iR}$, and the difference in means between responders and non-responders for the classifier is

$$\Delta \bar{x} = \bar{x}_R - \bar{x}_{NR} = \sum_{i=1}^{k} a_i (\bar{x}_R - \bar{x}_{iNR}).$$

For dichotomous variables the mean value $\bar{X}_{iR}$ is the equal to the proportion, p_{iR}, of responders for whom $x_i = 1$, and mean value $\bar{X}_{iNR}$ is the equal to the proportion, p_{iNR}, of non-responders for whom $x_i = 1$. Then

$$\Delta \bar{x} = \sum_{i=1}^{k} a_i (p_R - p_{iNR}) \equiv \sum_{i=1}^{k} a_i b_i,$$

for constants, b_i, derived from pilot data.

Let V(X) = the variance of random variable, X, and let us choose the a_i so that $S^2 = (\Delta\bar{x})^2 / V(\Delta\bar{x})$ is maximized as a measure of the separation of classes NR and R in the x-domain. Here the variance estimate from the given pilot data representing n_{NR} non-responders and n_R responders to experimental therapy (with independent x_i) is

$$\hat{V}(\Delta x) = \sum_{i=1}^{k} a_i^2 \left[\frac{p_{iNR}(1 - p_{iNR})}{n_{NR}} + \frac{p_{iR}(1 - p_{iR})}{n_R} \right] \equiv \sum_{i=1}^{k} a_i^2 c_i ,$$

for constants, c_i, derived from pilot data. Hence, using the estimate for the variance in the denominator,

$$S^2 \approx \frac{\left[\sum_{i=1}^{k} b_i c_i \right]^2}{\sum_{i=1}^{k} a_i^2 c_i} .$$

To maximize (or minimize) S^2 in the $a_1, a_2, \ldots a_k$ domain, we can solve the set of normal equations $\delta S^2/\delta a_1 = 0$, $\delta S^2/\delta a_2 = 0, \ldots \delta S^2/\delta a_1 = 0$ obtained by setting the partial derivatives equal to zero, where for any particular dichotomous variable, i,

$$\frac{\delta S^2}{\delta a_i} = 0 \approx \frac{2 b_i \sum_{i=1}^{k} a_i b_i}{\sum_{i=1}^{k} a_i^2 c_i} - \frac{2 a_i c_i \left[\sum_{i=1}^{k} a_i b_i \right]^2}{\left[\sum_{i=1}^{k} a_i^2 c_i \right]^2}$$

or

$$b_i - \frac{a_i c_i \cdot \left[\sum_{i=1}^{k} a_i b_i\right]}{\sum_{i=1}^{k} a_i^2 c_i} \approx 0 \quad \text{for } i = 1, 2, \ldots k,$$

which gives a set of k equations with k unknown variables, a_i, and 2k known variables, b_i and c_i, derived from the pilot data.

Two solutions are evident from simple inspection of the forgoing normal equations. Trivially, if $b_i = 0$ for all i, that is if $p_{iR} = p_{iNR}$, then we have a minimum with $S^2 = (\Delta\bar{x})^2/V(\Delta\bar{x}) = 0$. However, if $a_i = b_i = p_{iR} - p_{iNR}$, and if $c_i \approx c$, a constant (as is reasonable from inspection of the expression for the variance of proportions not too close to zero or one), we have an approximate solution to the normal equations for a maximum S^2, given the $b_i \neq 0$ and $c_i \neq 0$ from the training data. Thus we can expect roughly maximal separation of populations NR and R in the x domain if

$$a_i = b_i = p_{iR} - p_{iNR},$$

the differences in proportions of responders *vs* non-responders having dichotomous variable scores $x_i = 1$.

Although we assume that the x_i are poorly correlated, it can be shown numerically that this choice of the a_i is insensitive to small inter-correlations between predictors, x. If two predictors are strongly correlated, they can be combined into a single predictor, for example, high serum creatinine and high blood urea nitrogen can be lumped as "renal insufficiency", reducing the number of dimensions, k. Lumping highly correlated parameters in this way can improve separation of the classes NR and R and can avoid undesired over-weighting of the property measured by both correlated variables.

COMMENTS

Background

Clinical trials are too costly and take too long to complete. High costs of clinical trials add significantly to the ultimate costs of new medicines and medical devices. Delay in completion of a trial due to inefficient trial design can postpone, sometimes indefinitely, the transfer of promising new therapies from bench to bedside.

Research frontiers

The treatment of cancer, in particular, is moving towards the use of more specific therapies that are targeted to each tumor type. To facilitate this shift, tests are being developed to link specific genetic variations to specific drug effects using biomarkers that help predict how a given individual will respond to a drug.

Innovations and breakthroughs

This paper demonstrates how one can use biomarkers and other patient characteristics from a one-armed Phase II study or early-stage pilot data from an adaptive trail design to create a suitable classifier for discriminating responders from non-responders to a test drug or treatment.

Applications

Sample calculations using reconstructed raw data for a study of a novel drug treatment for lung cancer demonstrate that the choice of inclusion criteria can have a profound effect on the outcome of a clinical trial and that adjustment of inclusion criteria by quantitative means can produce protocols that achieve more with less. This example shows, using a model-based approach, how to minimize the time and cost of a clinical trial by selection of optimal patient inclusion criteria. Clear cost minimums exist for realistic scenarios with potential cost savings in millions of dollars. As the response rate for controls approaches 50%, the proper choice of inclusion criteria can mean the difference between a successful trial and a failed trial, no matter what the cost.

Terminology

Adaptive trial design: a clinical trial design that allows modification of aspects of the trial as it continues, based upon accumulating data in a statistically and intellectually valid way. Type II statistical error: failure to reject the null hypothesis when it is false, that is, a false negative interpretation of a research study.

Peer review

The topic is novel and one that is much welcomed in this space. The thinking is in the right direction.

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SYSTEMATIC REVIEWS

End points of clinical trials in metastatic castration-resistant prostate cancer: A systematic review

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Abstract

AIM: To review the definition and performance of the commonly used end points in trials of systemic therapies in metastatic castration-resistant prostate cancer patients.

METHODS: A literature search was undertaken on PubMed database to identify studies meeting established criteria, with the aim of selecting randomized clinical trials and study definition and performance of their end points. The end points were grouped into three categories: overall survival (OS), time-to-event end points, and response end points. A special analysis was performed for secondary end points of the studies which documented a benefit in OS in the experimental arm. Finally, publishes analyses for surrogacy of the included end points were also reported.

RESULTS: OS, time-to-event and response end points in 31 selected trials were analyzed. OS was the primary end point in 14 trials, and the secondary end point in 17. A time-to-event end point was the primary end point in 8 studies, and the secondary end point in 22; the most reported time-to-event end points were composite end points, and the events changed among trials. A response end point was the primary end point in 9 studies, in 3 it was prostate-specific antigen (PSA)-related, in 3 pain-related and in 3 mixed. A response end point was the secondary end point in 19 studies: PSA response and radiologic response were the most frequently used secondary end points in 19 and 11 trials, respectively, while pain response was used in 5 studies.

CONCLUSION: A homogeneous definition of progression in future trials is mandatory. Among response end points, pain-response and PSA-response appear to be the most reliable.

Key words: Metastatic castration-resistant prostate cancer; End points; Progression-free survival; Prostate-specific antigen; Chemotherapy; Palliative response

Core tip: The approval in the last decade of new drugs that have increased survival of patients with metastatic castration-resistant prostate cancer (mCRPC) has weakened the role of overall survival (OS) as end point. The prevailing bone-only spread of mCRPC severely limits disease evaluation using the standard criteria of conventional radiology. On the other hand, recent retrospective analyses of prostate-specific antigen response after chemotherapy did not support this measure as a surrogate end point of OS. This lack of reliable surrogate end points is a problem for the conduction of phase Ⅱ studies which test the activity of new drugs.

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INTRODUCTION

Prostate cancer (PC) is the most common malignancy in elderly men and the second leading cause of cancer death in Europe[1]. Death due to PC occurs in the later stages of the disease course in metastatic hormone-sensitive and mainly in metastatic castration-resistant prostate cancer (mCRPC)[2]. The median time from diagnosis of metastasis to death is 5 years[3].

Since 1996 a role for systemic antineoplastic treatment has been recognized, when a clinical trial, for the first time, reported a reduction in disease-related pain in patients undergoing cytotoxic chemotherapy with mitoxantrone and prednisone[4]. Subsequent studies have documented a benefit in overall survival (OS) for patients receiving systemic therapy with docetaxel[5,6], cabazitaxel[7], abiraterone[8], enzalutamide[9] and radium-223[10].

Due to the prevalent bone dissemination of mCRPC, metastatic disease is difficult to measure and to evaluate using the response evaluation criteria in solid tumors (RECIST)[11]. Therefore, the study of surrogate endpoints (SEs) of OS and the development of combined criteria for disease assessment were strongly supported by the prostate specific antigen working group (PSAWG)[12]. However, the prostate specific antigen (PSA)-response after first-line chemotherapy has not received validation as a SE of OS[13]. It lacks prospective validation[14,15], and the analysis of data from the TAX327 study reported wide confidence intervals around the estimate of PSA decline > 30%[14]. Similarly, PSA-response failed to be a SE of OS even after second-line chemotherapy[16].

As a result, the time-to-event measures have attracted the attention of researchers as possible SEs of OS, as they were reported by the Prostate Cancer Working Group 2 (PCWG2) in 2008, and are summarized in Table 1[17]. However, none of these measures, to date, has been validated as a SE of OS. Parallel to the increase in the number of effective drugs for mCRPC and the increase in median OS of patients, in the last decade there has been a rapid evolution of possible end points related to the disease and the patient.

The purpose of this systematic review is to examine the end points of randomized prospective studies of systemic treatments in mCRPC patients.

MATERIALS AND METHODS

A literature search of randomized trials of systemic treatments in patients with mCRPC was undertaken in October 2013. This search was performed on the electronic database PubMed. The criteria used for the search were as follows: "prostate cancer" and ("castration resistant" or "hormone refractory" or "hormone resistant") and ("chemotherapy" or "docetaxel" or "mitoxantrone" or "cabazitaxel" or "estramustine" or "radium" or "abiraterone" or "enzalutamide" or "prednisone" or "hydrocortisone" or "zoledronate").

The search was restricted to randomized prospective studies published from September 1993 to September 2013. Editorials, commentaries, letters, academic papers and abstracts were excluded, reviews were considered for references, but were not included in the final analysis, as well as other non-randomized studies. The participants were adult patients with a diagnosis of mCRPC who received systemic treatment, hormonal, cytotoxic or radiometabolic. A first selection of eligible studies from PubMed was performed independently by two authors (Colloca G and Governato I), who selected, by title and abstract, randomized studies that included patients with mCRPC and reported the analysis of one or more end points. Candidate articles were then selected for eventual inclusion in the review. Further literature was identified from the references lists of these articles. Differences of opinion were resolved by discussion or by the third review author (Venturino A).

This was followed by a further analysis of the selected articles, and only those that examined OS and at least one end point were included in the final analysis. The end points were grouped into three categories: overall survival, time-to-event end points, and response end points. Results of clinical trials and analyses for surrogacy of the included end points were discussed. A special analysis was performed for secondary end points in the 6 studies that documented a benefit in OS in the experimental arm. When the final report of a study did not supply adequate data on the secondary end points, further details were sought in the original protocol of the study, sometimes provided as an appendix on the journal's website, or from abstracts, comments and revisions, available at other websites.

RESULTS

From 1819 reports in PubMed, and subsequent evaluation of the reference lists of selected papers, 69 candidate articles were identified.

Following analysis and selection of manuscripts, 41 eligible articles were identified, which referred to the results of 31 prospective randomized studies. These studies were included in the analysis.

OS

OS was the primary end point in 14 trials, as summarized in Table 2. OS was reported as a secondary end point in 17 studies. OS was uniquely defined as the time from randomization to death from any cause. All six studies that changed the standard of care of mCPRC patients had OS as the primary end point.

Time- to-event end points

A time-to-event end point was the primary end point in 8 studies, as shown in Table 3. Only two studies reported a

Table 1 Prostate cancer working group-2 suggested outcome measures for metastatic castration-resistant prostate cancer trials

Variable	Outcome measure
Progression criteria	
PSA	A favorable effect on PSA may be delayed for 12 wk or more, even for a cytotoxic drug Decline from baseline. PSA increase ≥ 25% and ≥ 2 ng/mL above the nadir, and which is confirmed by a second value 3 or more weeks later No decline from baseline. PSA progression ≥ 25% and ≥ 2 ng/mL after 12 wk
Bone metastases	The appearance of ≥ 2 new lesions, and, for the first reassessment only, a confirmatory scan performed 6 or more weeks later that shows a minimum of 2 or more additional new lesions. The date of progression is the date of the first scan showing the changes
Soft tissue lesions	RECIST criteria, with the additional requirement that progression at first assessment is confirmed by a second scan 6 or more weeks later. For some treatments, a lesion may increase in size before it decreases
Symptoms	Consider independently of other outcome measures. Document pain/analgesia at entry and measure repeatedly at 3- to 4-wk intervals. Ignore early changes (≤ 12 wk) in pain or HRQOL in the absence of compelling evidence of disease progression. Confirm progression of pain or HRQOL end points ≥ 3 wk later
Response criteria	
PSA	Ignore early rises (prior to 12 wk) in determining PSA response Record the percent change from baseline at 12 wk, and the maximal change at any time using a waterfall plot
Bone metastases	Record outcome as new lesions or no new lesions. If at the first scheduled reassessment there are not new lesions, continue therapy; if there are new lesions, perform a confirmatory scan 6 or more weeks later and only where new lesions are present stop therapy due to progressive disease
Soft tissue lesions	RECIST criteria, with some caveats: only report changes in lymph nodes that were ≥ 2 cm in diameter at baseline; record changes in nodal and visceral soft tissue sites separately; record complete elimination of disease at any site separately; confirm favorable change with second scan; record changes using a waterfall plot
Symptoms	Consider independently of other outcome measures. Document pain/analgesia at entry and measure repeatedly at 3- to 4-wk intervals. Ignore early changes (≤ 12 wk) in pain or HRQOL in the absence of compelling evidence of disease progression Confirm response of pain or HRQOL end points ≥ 3 wk later

PSA: Prostate-specific antigen; RECIST: Response evaluation criteria in solid tumors; HRQOL: Health-related quality of life.

significant benefit in the experimental arm, documenting the effect of treatment with mitoxantrone or vinorelbine in patients with mCRPC: the USOR study documented a time-to-treatment failure (TTTF) of 8.1 mo *vs* 4.1 mo for the mitoxantrone and prednisone arm *vs* prednisone alone[28]; another study reported a significantly higher progression-free survival (PFS) of 3.7 mo *vs* 2.8 mo for the combination of vinorelbine plus hydrocortisone *vs* the hydrocortisone arm[29].

Time-to-progression (TTP) was the primary end point in 3 studies, PFS in 2, TTTF in 2, and time-to-subjective-progression in another trial. The definition of events related to progression varied between trials. In the EORTC trial, TTP was evaluated relative to the best condition, observed at the start of treatment or obtained during treatment; this occurred if patients had an increase in pain score by at least one level, increase in the daily analgesic dose by at least 25%, the need for additional pain treatment, and WHO performance status deterioration by at least one level[27]. In the Sipuleucel-T study, TTP was defined as progressive disease on serial radiographic imaging tests, new cancer-related pain associated with a radiographic anatomic correlation, or other clinic events consistent with progression (spinal cord compression, nerve root compression, pathologic fracture)[30]. In the Atrasentan trial, TTP was determined according to radiographic (different criteria to define progression of bone lesions and soft-tissue lesions) and clinical measures (metastatic pain, skeletal-related events, requirement of a new intervention)[31]. PFS was also a composite end point in the vinorelbine trial, in which it was defined as the time from randomization until progression or death, last news or initiation of a new therapy; the event of progression was the first among radiologic, serologic or pain-related events[29]. PFS in the PROSTVAC trial was related to radiologic progression by RECIST criteria or to the appearance of at least two new lesions on a bone scan, even if patients who developed clinical signs or symptoms of progression, but who did not meet the radiologic criteria were also considered to have progressed at the discretion of the investigator[32]. TTTF was the primary end point in two positive trials: in the USOR trial it was an aggregate end point, defined as the interval between the start of treatment and occurrence of progressive disease, removal from study or initiation of other antitumor therapy; in this study, progressive disease was radiologic only, by RECIST criteria[28]; the PROSTY study used a different definition of TTTF, calculated from randomization to first disease progression (PSA or measurable), unacceptable toxic effects, death or discontinuation of therapy for any reason[33].

Time-to-event end points were reported as secondary end points in 22 studies. In Table 4 these studies are grouped according to the event that defined the measure. The events most often used to define the end point were radiologic progression and PSA progression, in 7 and 6 studies, respectively, although 9 studies used a composite end point of progression. A "subjective" progression was the primary end point in the DAPROCA 9002 study[26], and was defined as an increase in a score including can-

Table 2 Characteristics of randomized clinical trials including overall survival as the primary end point after first-line and/or second-line medical treatment of metastatic castration-resistant prostate cancer

Trial	Arms	No. pts	Publication year	Main conclusion	Median OS
LSG Trial[18]	LIA	160	1998	After adjustment for baseline prognostic factors, HR for OS favored the first arm	10.3 mo
	CPA	161			10.3 mo
CALGB 9182[19]	MXN + HDC	119	1999	Better PSA-RR and PFS in the first arm	12.3 mo
	HDC	123			12.6 mo
HOG/FNC Trial[20]	VBL + ESM	95	1999	Better PSA-RR and PFS in the first arm	11.9 mo
	VBL	98			9.2 mo
SWOG 9916[6]	DOC + ESM	338	2004	Longer OS in the first arm	17.5[1] mo
	MXN + PDN	336			15.6 mo
TAX327[5]	MXN + PDN	337	2004	DOC-based chemotherapy is the new standard first-line treatment of mCRPC	16.5 mo
	DOC + PDN	335			18.9[1] mo
	wDOC + PDN	334			17.4 mo
[3]SPARC[21]	SPT + PDN	635	2009	Better PSA-RR in the first arm	61.3 wk
	Placebo + PDN	315			61.4 wk
[2]TROPIC[7]	CBZ + PDN	378	2010	CBZ-based therapy is effective in mCRPC progressing to DOC	15.1[1] mo
	MXN + PDN	377			12.7 mo
IMPACT[22]	SIP-T	341	2010	Similar results	25.8[1] mo
	Placebo	171			21.7 mo
[2]COU-AA-301[8]	ABI + PDN	797	2011	ABI hormonal therapy is effective in mCRPC progressing to DOC	14.8[1] mo
	Placebo + PDN	398			10.9 mo
ASCENT-2[23]	DOC + DN101	477	2011	DN101 is inferior to PDN	17.8 mo
	DOC + PDN	476			20.2[1] mo
CALGB 90401[24]	DOC + PDN + BEV	524	2012	Better PSA-RR and PFS in the first arm	22.6 mo
	DOC + PDN + Placeb	526			21.5 mo
[2]AFFIRM[9]	ENZ	800	2012	ENZ hormonal therapy is effective in mCRPC progressing to DOC	18.4[1] mo
	Placebo	399			13.6 mo
ENTHUSE M1C[25]	DOC + ZBT	524	2013	Similar results	20.0 mo
	DOC + Placebo	528			19.2 mo
[3]ALSYMPCA[10]	Radium-223	614	2013	Radium-223 effective in mCRPC with painful bone metastases	14.9[1] mo
	Placebo	307			11.3 mo

[1]Statistically significant difference; [2]Trial of second-line medical treatment; [3]Trial of first or second-line medical treatment. ABI: Abiraterone; BEV: Bevacizumab; CBZ: Cabazitaxel; CPA: Cyproterone acetate; DN101: High-dose calcitriol; DOC: Docetaxel; ENZ: Enzalutamide; ESM: Estramustine; HDC: Hydrocortisone; HR: Hazard ratio; LIA: Liarozole; mCRPC: Metastatic castration-resistant prostate cancer; MXN: Mitoxantrone; OS: Overall survival; PDN: Prednisone; PFS: Progression-free survival; PSA-RR: Prostate-specific antigen response rate; SIP-T: Sipuleucel-T; SPT: Satraplatin; VBL: Vinblastine; wDOC: Weekly-docetaxel; ZBT: Zibotentan.

cer-related weight loss of more than 10%, cancer related decrease in hemoglobin of at least 25%, or the need for at least 2 units of blood a month. A similar composite end point of clinical progression was present in the ECOG 3882 study[34].

Disease progression has had a very variable definition in studies of mCRPC. In the SWOG 99-16, progression was defined as the occurrence of a 50% increase or an increase of 10 cm^2 in the sum of measurements of metastatic lesions over the sum at baseline, reappearance of any lesion that had disappeared, appearance of any new lesion or death[6]. A more recent study, the SPARC study, calculated PFS as the time from randomization to the first occurrence of tumor progression, skeletal-related events, symptomatic progression, or death from any cause[21], while the definition in the TROPIC study also included PSA progression[7]. Finally, progression has very often been defined by mixed criteria, either as a radiographic event according to RECIST criteria or scintigraphic progression, often the appearance of two or more new lesions on a bone scan, or as clinical progression or serological progression, and the first of these events is considered as the decisive event to define the date of progression.

Treatment failure has been reported as a composite outcome, which usually includes the first event in the progression of disease, the occurrence of unacceptable toxicity, patient refusal to continue therapy, removal from the study or the beginning of another antineoplastic treatment. Time to treatment failure was reported as the primary end point in 2 trials[31,33].

Response end points

A response end point was the primary end point in 9 studies, as summarized in Table 5 and 6. The end point was a palliative response in three studies, a PSA-response in 2, a double response (PSA and radiologic) in one, and a mixed response in the other 3 trials. Only two studies showed a positive result in the experimental arm: the ECOG 3882 study reported a higher mixed response rate, 63% *vs* 27% for diethylstilbestrol plus doxorubicin arm *vs* doxorubicin[34], and the CALGB 9583 trial documented a higher mixed response rate after antiandrogen withdrawal in the ketaconazole plus hydrocortisone arm[41]. In more recent studies, the PSA response rate did not document significant differences when used as a primary end point[38,39].

The CALGB 9181 study provided a mixed response

Table 3 Characteristics of randomized clinical trials including time-to-event measures as the primary end point after first-line medical treatment of metastatic castration-resistant prostate cancer

Trial	Arms	No. pts	Publication year	End point	Result (mo)
DAPROCA 9002[26]	ESM	61	1997	TTSP	2.2
	Placebo	68			5.0
EORTC[27]	FLT	100	2001	TTP	2.3
	PDN	101			3.4
USOR[28]	MXN + PDN	56	2002	TTTF	8.1[1]
	PDN	63			4.1
Vinorelbine Trial[29]	VNR + HDC	206	2004	PFS	3.7[1]
	HDC	208			2.8
UCSF[30]	SIP-T	82	2006	TTP	11.7
	Placebo	45			10.0
Atrasentan Trial[31]	ATR	408	2007	TTP	HR = 0.89
	Placebo	401			
PROSTVAC[32]	PROSTVAC	82	2010	PFS	3.8
	Placebo	40			3.7
PROSTY Trial[33]	DOC three-weekly	184	2013	TTTF	4.9
	DOC two-weekly	177			5.6[1]

[1]Statistically significant difference. ATR: Atrasentan; DOC: Docetaxel; ESM: Estramustine; FLT: Flutamide; HDC: Hydrocortisone; HR: Hazard ratio; MXN: Mitoxantrone; PDN: Prednisone; PFS: Progression-free survival; PROSTVAC: Vaccinia-PSA-TRICOM and Fowlpoc-PSA-TRICOM; SIP-T: Sipuleucel-T; TTP: Time to progression; TTSP: Time to subjective progression; TTTF: Time to treatment failure; VNR: Vinorelbine.

Table 4 Disease-progression-related events used to define secondary end points in randomized trials of metastatic castration-resistant prostate cancer

Progression event	Trial number	Ref.
Clinical	2	[26,34]
Pain	2	[25,35]
Skeletal-related events	4	[8-10,23]
Radiological	7	[6,8,9,22,24,25,36]
Prostate-specific antigen	6	[8-10,24,25,31]
Alkaline phosphatase	2	[10,31]
Mixed	9	[7,19,20,21,28,33,37-39]

end point, which included the classic radiological criteria, when applicable, PSA response and bone scan evaluation, but the latter was used only to define progression[40]. Two distinct response end points, radiologic response and PSA response, were the primary end points in the INT 0159 study[36]. In the ECOG 3882 trial, all patients were periodically evaluated by bone scan, serum acid phosphatase, and clinical status. There were reported internal criteria in the study to define the scintigraphic response and the serological response of acid phosphatase; a clinical response derived from the reduction of a point from baseline on the ECOG scale, an increase of 5% in weight, or an increase in hemoglobin of 2 g for at least 3 mo. In this study a scale of priorities was formalized to define progression: in patients with radiologically measurable disease, RECIST criteria were applied, and only in the case of radiologic stability a worsening of bone scintigraphy

Table 5 Characteristics of randomized clinical trials including radiologic or serological response measures as the primary end point after first line medical treatment of metastatic castration-resistant prostate cancer

Trial	Arms	No. pts	Publication year	Response-related outcome	Response rate (%)
CALGB 9181[40]	MA 160 mg/die	73	2000	MRR	3%
	MA 640 mg/die	76			3%
INT 0159[36]	SUR 3,1	128	2002	PSA-RR/	24%/9%
	SUR 5,3	124		RRR	28%/7%
	SUR 7,6	120			34%/15%
ECOG 3882[34]	DOXO + DES	74	2003	MRR	63%[1]
	DOXO	76			27%
CALGB 9583[41]	AWD + KET + HDC	128	2004	MRR	27%[1]
	AWD	132			11%
Belgian Trial[38]	DOC + PDN + ESM	71	2008	PSA-RR	41%
	DOC + PDN	69			25%
NHS Trial[39]	DEX + ASP + DES	136	2011	PSA-RR	64%
	DEX + ASP	133			68%

[1]Statistically significant difference. ASP: Aspirin; AWD: Anti-androgen withdrawal; DES: Diethylstilbestrol; DEX: Dexamethasone; DOXO: Doxorubicin; ESM: Estramustine; HDC: Hydrocortisone; KET: Ketoconazole; MA: Medroxyprogesterone acetate; MRR: Mixed response rate; PDN: Prednisone; PSA-RR: Prostate-specific antigen response rate; RRR: Radiologic response rate; SUR: Suramine.

Table 6 Characteristics of randomized clinical trials including clinical response measures as the primary end point after first line medical treatment of metastatic castration-resistant prostate cancer

Trial	Arms	No. pts	Publication year	PRO measure	Palliative response rate (%)
Canadian Trial[4]	MXN + PDN	80	1996	Palliative response (moore)	29[1]
	PDN	81			12
SIG-1 Trial[37]	SUR + HDC	228	2000	Palliative response (BPI + analgesics)	43[1]
	Placebo + HDC	230			28
NCIC PR06[35]	MXN + PDN + CLD	104	2003	Palliative response (moore)	46
	MXN + PDN + Placebo	105			39

[1]Statistically significant difference. BPI: Brief pain inventory; CLD: Clodronate; HDC: Hydrocortisone; MXN: Mitoxantrone; PDN: Prednisone; PRO: Patient-reported outcome; SUR: Suramine.

or of clinical status were sufficient criteria for assigning progression[34]. In the CALGB 9583 study, patients with measurable disease were considered responsive only if they reported a reduction of at least 50% in the sum of the products of the target lesions, but this was associated with a reduction in baseline PSA of 75% and confirmed after 2 wk[41].

Response end points were also used as secondary end points in 19 studies, as reported in Table 7. PSA response

Table 7 Disease-control/response related events used to define secondary end points in randomized trials of metastatic castration-resistant prostate cancer

Progression event	Trial number	Ref.
Clinical	1	[27]
Pain	5	[7-9,18,21]
Radiological	11	[4,5,7,19,20,21,24,26,28,37,38]
Prostate-specific antigen	19	[4,5,7,8,19-22,24,25,27-29,33,35, 37,38,40,41]
Alkaline phosphatase	1	[10]
Immunity	1	[32]

and radiologic response were the most commonly used secondary end points in 19 and 11 trials, respectively, while pain response was used in 5 studies.

Even though surrogacy has not been validated in prospective studies, more recent studies have continued to report the prostate-specific antigen response rate (PSA-RR) only as a primary end point, defining it as a reduction > 50%, as recommended by the previous PSAWG criteria[38,39,41].

Other response measures were derived from the assessment of disease-related symptoms, such as pain or fatigue, and other patient-related outcomes. Table 6 summarizes the three studies in which the primary end point was clinical and correlated with the response. Although these studies were published between 1996 and 2003, even recent studies have evaluated clinical outcomes, largely related to the patient, as secondary end points, better defining their thresholds of response. In the Canadian study[4], the TAX 327[5], SWOG 99-16[42], SPARC[21], TROPIC[7], and NCIC PR06[35], the palliative response was defined as a 2-point decrease in pain as assessed by a 6-point scale, patient-reported present pain intensity (PPI), without an increase in analgesic medication, and maintained for two consecutive evaluations at least 3 wk apart; pain progression required an increase in the weekly average PPI score of ≥ 1 point from baseline or ≥ 2 points from nadir for ≥ 2 consecutive wk or a more than 25% increase from baseline in the weekly average analgesic score for ≥ 2 consecutive weeks[43]. In the studies COU-AA-301[8], AFFIRM[9], and SIG-1[37] intended for pain palliation, a 30% decrease in the brief pain inventory-short form (BPI-SF) score in the absence of an increase in analgesic usage, while pain progression was an increase of > 30% in the worst pain in the past 24 h on the BFS observed on two consecutive evaluations 4 wk apart without a decrease in analgesic usage score, or an increase in analgesic usage score > 30% observed at 2 consecutive evaluations 4 wk apart; the timing of pain response assessment was reported at week 13 in the AFFIRM trial[9]. Other secondary end points were often protocol-dependent and included the Newling palliative response in the LSG trial[18], with measures of pain and analgesic usage scales, the subjective response in the EORTC trial[27], with pain and performance status scores, or the subjective response in the DAPROCA 9002 trial[26], including pain, analgesics use, steroids, radiotherapy and performance status, and finally the clinical response in the ECOG 3882 study, which collected performance status, pain, analgesic, hemoglobin and body weight scores[34]. Pain response was confirmed as highly predictive of outcome in patients with mCRPC[44], but was weaker than the PSA decline > 30% as a possible SE of OS[14].

Unlike PSA response and pain response, response using quality of life (QoL) scores did not correlate with OS in the TAX327 trial[45]. This study evaluated a QoL-response using the FACT-P questionnaire: a maximum score of 156 points indicated the highest level of QoL measured by FACT-P, and QoL improvement was defined by ≥ 10% (16 points) increase maintained for at least 3 wk; inversely, a decrease of 16 points was considered a deterioration in QoL. Among patients enrolled in the trial an impairment of QoL was detected in 92% of patients with pain and in 75% of those without pain, suggesting that disease-related symptoms other than pain contribute to QoL. The complexity of the clinical situation is underlined by the fact that patients with minimal symptoms at the beginning of chemotherapy in the weekly docetaxel arm most likely had an initial worsening of QoL scores. Therefore, it was not surprising that different from pain-response and PSA-response, QoL-response did not predict OS[45].

Few studies have defined the role of other symptoms. Of these the most common is fatigue. In the COU-AA-301 trial, an analysis of a patient-reported questionnaire, the BFI, was performed. After a baseline assessment of the fatigue score distribution, some clinically significant changes in fatigue intensity and fatigue interference were defined. It appeared that in the abiraterone plus prednisone arm more patients experienced an improvement in fatigue intensity and fatigue interference and a delay in the progression of both[46].

DISCUSSION

PCWG2 recommended that, when possible, all assessments of the disease are carried out at the same time interval. In addition to PSA changes, it is important to confirm the post-treatment changes in the measurable target lesions, those of the radionuclide bone scan, and those of symptoms[17]. Following the results of studies on immunotherapy and targeted therapy in mCRPC the opportunity of an appropriate definition of different end points was discussed, and these were defined according to the different drugs and their mechanisms of action. It was proposed that the assessment of cytotoxic and non-cytotoxic therapies is performed in a different way, as cytotoxic chemotherapy and hormonal therapy produce a reduction in PSA and a regression of target lesions, while measures of early response to treatment would not show the effect of immunotherapy and some targeted therapies. In our opinion, although it is difficult to obtain evidence of a surrogate end point according to the criteria of Prentice[13], it is nevertheless appropriate that the search for new SEs should avoid further fragmentation related to the different treatment options.

Potential biases in the present review could be due to the fact that we restricted the included studies to randomized clinical trials; these trials define the best level of evidence available, but this restriction could have excluded many other possible secondary end points. It is also possible that the population in the included studies was heterogeneous: this is because in some studies a second-line treatment was assessed, while in others patients with serologic progression only, with an early stage of mCRPC, were enrolled. Finally, the possibility of publication bias, *i.e.*, studies that did not find the treatment to be effective may not have been published.

To date, none of the end points studied in mCRPC has sufficient evidence to be considered a valid SE of OS. However, many end points have been well standardized and may allow us to better compare the results between studies. Following the recommendations of PCWG2, the time-to-event end points have received increasing attention compared to the response end points.

PFS is usually defined as the time from randomization to the first event in clinical, radiological or biochemical progression or death. PFS has been debated as a SE of OS in studies of mCRPC. The serological progression was the first event in disease progression in 60% of cases, bone progression in 18%, radiological progression in 7%, and death was the first event in 15%. Therefore, in phase-2 studies a PSA-related and time-to-event end point appears to be more practical than any other response end point, and may allow easier comparison of the results between studies with respect to the progression end points. Some preliminary information on PSA kinetics measures, as a growth rate constant, suggest that new measures related to PSA could better predict OS regardless of the type of medical therapy, and promises to overcome many of the limitations of PSA response/progression as defined by PCWG2[47].

On the other hand, clinical research on patient-reported instruments is producing encouraging results and the use of patient-reported outcomes (PROs) in clinical trials is recommended[48]. PRO is any outcome based on data provided by patients or patient proxy, as opposed to data provided by other sources. Several types of measures have been included in PROs, such as QoL, functional status, symptoms, overall well-being, satisfaction with care, and treatment adherence. The predictive value of PROs-related end points in cancer patients has been reported in a comprehensive clinical review[49]: in 36/39 selected trials at least one PRO was significantly associated with survival after multivariate analysis. Pain-related outcomes have been well studied in patients with mCRPC and measured by two well-defined response end points, but their application is limited to patients with disease-related pain; they appear to be good candidate end points in trials of late stage mCRPC. Similar palliative fatigue-related end points could soon be developed. In addition to pain and fatigue, a rigorous evaluation of the general QoL and its domains may allow us to better capture the clinical benefit of new drugs and could address decision-making. FACT-P and EORTC-QLQ-PR25 are two questionnaires that have been extensively studied in patients with prostate cancer. While the former seems more detailed[50], it is always better to make a direct comparison with the aim of defining a unique tool for assessing QoL. A final standardization of this instrument could allow us to compare more information between studies.

In conclusion, given the failure of all end points to act as surrogates for OS in the mCRPC setting, defining new PSA-related and patient-related end points and standardizing them remain the most important goal of clinical research. To accomplish this, additional retrospective assessments of recent prospective studies with the aim to test new parameters related to PSA-kinetics and to extrapolate other scores from QoL questionnaires can help identify new end points as surrogates.

COMMENTS

Background

The prevalent bone-only dissemination of metastatic castration-resistant prostate cancer (mCRPC), limits the application of commonly used radiologic criteria of progression and response in the assessment of new drugs. Prostate-specific antigen (PSA)-related criteria of response/progression are not effective surrogate end points of overall survival and failed to predict the efficacy of immunotherapy and of some targeted therapies.

Innovations and breakthroughs

In this review article the authors report how the end points of randomized trials of mCRPC change with the introduction of new drugs and with the improvement of disease-related symptoms and overall survival. They identify two future key areas in the field of end point research of mCRPC, PSA kinetics and patient-reported outcomes.

Applications

To date, phase-2 trials of new anticancer drugs in mCRPC patients require a PSA-related and time-to-event end point. However, new end points should be tested prospectively.

Peer review

This manuscript aims to systematically review the end points of 31 prospective clinical trials of medical therapies and/or radiomethabolic treatments in hormonal-refractory prostate cancer.

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EDITORIAL

Effects of physical activity in Parkinson's disease: A new tool for rehabilitation

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Abstract

Parkinson's disease (PD) is a common neurodegenerative disease characterized by bradykinesia, tremor, rigidity, and postural instability. Motor disorders are composite and combined, adversely affecting the patient's health. Tremor and rigidity are correlated with worsening manual dexterity as well as postural changes such as akinesia and camptocormia. Moreover, gait alteration as well as postural instability, with consequent impairment in balance, increase the risk of falls. It is well known that these symptoms respond poorly to pharmacologic therapy in PD patients. Physical therapy is the most effective non-pharmacological aid to PD patients. Available data in the literature indicate that any rehabilitation protocol has to focus on: cognitive movement strategies, cueing strategies, and improved physical capacity and balance. Different training programs for PD patients have been designed and evaluated but only specific training strategies, tailored and individualized for each patient, may produce improvements in gait speed and stride length, decrease motor and balance symptoms and improve quality of life. Furthermore, aerobic training may improve muscle trophism, strength and mobility. It seems reasonable to state that tailored physical activity is a valid tool to be included in the therapeutic program of PD patients, considering that this approach may ameliorate the symptoms as well as the overall physical incapacity, reduce the risk of falls and injuries, and ultimately improve quality of life.

Key words: Parkinson's disease; Motor disorders; Postural instability; Physical exercise; Training

Core tip: A review of the literature underlines the importance of tailored physical activity in patients with Parkinson disease. Several studies demonstrated the key role that specific training strategies may have on motor disorders and postural instability affecting patients with Parkinson disease. Since it has been clearly demonstrated that these symptoms respond poorly to pharmacologic therapy, it seems necessary to combine the traditional treatment of Parkinson disease with a specific exercise training strategy in order to reduce motor disorders as well as postural instability, with the aim of improving quality of life of the patients affected by this neurologic disease.

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PARKINSON'S DISEASE

Parkinson's disease (PD) is the second most common neurodegenerative disorder, after Alzheimer's disease. PD affects 1% of the population over 60 years of age, and the risk increases proportionally with age.

The primary symptoms of PD are bradykinesia, trem-

or, rigidity, and postural instability. Bradykinesia refers to the slowness of the patient's movements[1] and affects every single patient[2]; it is evident especially when evaluating gait, but it affects every voluntary movement. Patients also manifest a global reduction in spontaneous movements (*e.g.*, gestures, winking) and deliberate movements (*e.g.*, arm swing), defined as akinesia[1]. PD's tremor affects 75% of the diagnosed subjects[3]. This "resting tremor" differs from essential tremor since it manifests when the body part affected is not involved in voluntary movements. Rigidity affects 90%-99% of PD patients[4] and it is caused by muscular hypertonia; the typical posture of PD patients, camptocormia, is in fact due to excessive activation of flexor muscles in the trunk and limbs[4]. Postural instability is "the impairment in balance that compromises the ability to maintain or change posture such as standing and walking"[5]. This condition does not usually affect the early stages of the illness, and is one of the most difficult symptoms to be treated[6].

For many reasons, it is essential to describe the impaired Parkinsonian gait. In chronological order, patients experience difficulties when starting to walk, a condition called "start hesitation"[7]; walking speed and stride length are abnormally reduced; lower limb joints excursion is reduced due to rigidity; timing of steps is extremely irregular and asymmetric; and arm swing is decreased or absent. Moreover, 2 typical features are also present: festinating gait or festination is characterized by the patient's sudden acceleration as an attempt to keep their center of gravity between their feet in order to compensate for their flexed posture[8]. Freezing of gait (FoG) is defined as a sudden stop in the patient's gait, often with legs trembling in place and the sensation of being "glued" to the floor. This symptom manifests more frequently while turning, when the path changes or gets more narrow, in a diagonal direction, when dealing with obstacles and other stressful situations or just before reaching the destination[8-11].

Subjects affected by PD also experience several non-motor symptoms, such as autonomic dysfunctions (dysphagia, constipation, urinary incontinence, sexual dysfunction, orthostatic hypotension) cognitive impairment, dementia, depression (which affects 30%-40% of patients)[12], anxiety, sleep disorders and decreased olfactory sense.

PD motor disorders are very complex and interconnected, and their adverse consequences for both patient's health and quality of life are very well known. Tremor and rigidity significantly reduce the quality of life since they worsen manual dexterity, thus affecting simple everyday activities such as cutting food pill-taking and posture[13]. Moreover, gait impairments (especially FoG and stride variability) as well as postural instability (and the consequent reduced balance) represents harmful conditions of PD since they increase the risk of falls[11,14,15]. According to statistics, almost 70% of patients experience falls at least once per year[16] and 25% of subjects in the first 10 years of disease suffer a hip fracture[11], an injury correlated with high morbidity and mortality in PD patients[17]. Furthermore, falls can trigger a vicious circle that aggravates even more the patient's condition: fear of other falls and injuries reduce the subjects' mobilization, which in turn causes sarcopenia, decreased fitness, osteoporosis, loss of independence, social isolation, and reduced participation in simple daily activities[11,18-20]. In extreme cases of these impairments there is a condition which is sometimes referred as "malignant parkinsonism", in which rapid disease progression, premature home nursing, depression and cognitive decline, increase the mortality risks[11].

There is no cure for PD and pharmacological treatment (of which levodopa is the long-term gold standard) still lacks significant effects on the previously described harmful symptoms. Indeed, postural instability, balance problems and gait disorders such as FoG and stride variability respond poorly to medication[9-11,21-24]. Even worse, many authors describe a "paradoxal effect" of medication[11,21,25] since two-thirds of falls occur when patients are under the effect of medication ("on" phase)[21]. This condition may be explained by the fact that dopaminergic treatment improves gait speed and general mobility (thus stimulating the patients to move), but not balance and postural instability[21,25]. Finally, some adverse effects of long-term pharmacological treatment, such as levodopa-induced dyskinesia and orthostatic hypotension, are very well known causes of falls[18,25,26].

Therefore, it is evident that the study of new approaches aimed to improve the motor aspects of PD patients is necessary. Nowadays, besides optimal medication, it is evident that physical therapy is the most effective non-pharmacological aid for patients with PD. It is not the aim of this paper to describe in detail the pathophysiology underlying each symptom, but putative mechanisms for each exercise protocol will be discussed in detail in the ensuing paragraphs.

EXERCISE GUIDELINES FOR PD

The most relevant guidelines for physical exercise in PD have been designed by Morris[27] and Keus *et al*[28] (Table 1).

External cues represent effective interventions aimed to improve motor performance, especially gait. Cognitive movement strategies refer to those mental techniques taught to patients in order to improve their everyday motor tasks. The key intervention is to teach the patient to subdivide complex and automated motor sequences in series of single, simple movements that must be performed in the correct, fixed order. This strategy is aimed to render motor performance as a conscious activity, thus avoiding dual-tasking[27,28] as well as bypassing the defective basal ganglia[28]. Balance training is another crucial point of the model, since it can prevent falls. Furthermore, improving physical capacity with aerobic training, strength and flexibility exercises may reduce symptoms as well as improve the patient's general well-being and quality of life.

In this setting, the individualization of training represents a crucial approach. Indeed, PD symptoms change, often with fluctuations, with the progression of the dis-

Table 1 Essential points in Parkinson's disease exercise therapy

	Effects
Cueing strategies	Improve motor performance (especially gait)
Cognitive movement strategies	Improve everyday motor tasks (walking, standing up, sitting down, dressing, *etc.*), and quality of life
Balance training	Prevent risk of falls, improve postural stability
Aerobic training	Improve physical capacity
Strength and flexibility	Improve general well-being and quality of life

ease. Therefore, physical therapists, in conjunction with the caregivers and neurologists, should adapt, modify and tailor the exercise program to the patient's specific needs. Regular assessments[28] should be performed in order to analyze the effects of both medications and physical therapy, and consequently adapt them to the patient's actual condition.

To achieve feasible improvements, a physical therapy protocol should be at least 8-10 wk long. Ideally, patients should train 3 d per week, 60-75 min per session. Moreover, it is also recommended to perform stretching exercises daily in order to reduce rigidity. Obviously, choosing an enjoyable and stimulating exercise protocol, thus promoting adherence and long-term compliance is of major importance when considering both the patient's prognosis and quality of life.

TRAINING STRATEGIES

Different training programs for patients with PD have been designed and evaluated.

Treadmill

Treadmill training is probably the most examined form of exercise for patients with PD.

Single-pulse Transcranial Magnetic Stimulation studies have shown abnormalities of the Cortical Silent Period (CSP duration) and other corticomotor excitability measures in patients with PD, reflecting greater corticomotor excitability in these patients. It is known that CSP is mainly mediated by gamma-aminobutyric acid (GABA)-B receptors, and abnormalities of GABAergic transmission are key points of the pathophysiology of movement disorders involving the basal ganglia. In addition, voluntary exercise may increase brain-derived neurotrophic factor (BDNF) levels thus enhancing neuronal function by promoting synaptogenesis and neurogenesis. Indeed, BDNF modulates the level of functional inhibition in an activity-dependent manner by regulating the number of GABAergic interneurons. While the role of BDNF in modulating GABA-mediated inhibitory transmission is not fully understood, conceivably the lengthening of CSP, related to high intensity exercise, may be related to an exercise-induced increase in BDNF[29].

The rationale behind the use of a treadmill in PD patients, proposes that the device works as an external cue[9,30-33] which bypasses the defective basal ganglia. In this setting patients do not need to pay attention to triggering[31,34,35], selecting and maintaining a motor sequence[35], and they can focus only on the motor action of walking[35]. Furthermore, as stated by Cakit *et al*[21], "a guiding principle in neurologic rehabilitation is that a skill will be improved if it is practiced"; therefore, walking on a treadmill can improve Parkinsonian gait since, as described by several authors[33,36,37], it can also generate motor learning. The need for improving gait and walking in people affected by PD is related to the achievement of performance improvement. In addition, gait training is necessary to reduce gait disorders (such as FoG, gait variability and festination) which directly cause reduced mobility, falls and injuries that can severely worsen the patient's health and quality of life. As described by Frenkel-Toledo *et al*[31], the rhythm imposed by the machine regularizes the patient's gait, with steps becoming longer and less variable. Considering that stride variability represents the main risk indicator of falls in the elderly[38], this improvement acquires even more significance. Indeed, different studies[21,36,39] show that treadmill training directly decreases risk and actual falls in subjects affected by PD.

As proven by 2 studies, even a single session of treadmill training generates improvements in the gait of PD patients. In the study of Pohl *et al*[32], patients tried 3 different interventions for gait treatment: Speed-Dependent Treadmill Training (SDTT, in which the patient walks 10 s at his/her maximum safe speed, and if that speed was sustainable it would have been increased after a short rest), Limited Progressive Treadmill Training (in which the belt speed was never increased over walking speed reported at baseline) and Conventional Gait Therapy (CGT, based on Proprioceptive Neuromuscular Fasciculation concepts). After just a 30-min training session, both of the treadmill protocols showed significant improvements in gait speed and stride length; also, double stance duration was decreased. Similarly, Bello *et al*[40] described improvements in gait speed and stride length after a single, 20-min treadmill session.

Several studies focused on the effects of a longer-term protocol in Parkinson's patients. Miyai *et al*[41] demonstrated that, in comparison with traditional physical therapy, a 4-wk body weight-supported treadmill training (BWSTT) program was effective on symptoms]as shown by the unified parkinson's disease rating scale (UPDRS)] as well as on gait speed. In another study of Miyai *et al*[37], patients received a 45-min BWSTT training 3 times a week for 1 mo, with belt speed progressively increased over the training period. Results showed higher gait speed and decreased number of steps over a 10 m distance, and effects lasted, respectively, at the 1- and 4-mo-follow up. Herman *et al*[36] demonstrated that an intensive treadmill protocol, with patients training 30 min, 4 times per week over a 6-wk period (with belt speed progressively increased), could generate improvements not only in gait parameters (speed, swing time variability), but also in balance, motor symptoms (as showed by the values of UPDRS-Ⅲ) and quality of life (assessed with Parkinson's Disease Questionnaire-39). Four studies evalu-

Table 2 Results of treadmill training

Pohl *et al*[32]	Single session 10 s at maximum safe speed	After 30 min training	Improvement in gait speed and stride length
Bello *et al*[40]	Single 20 min session		Improvement in gait speed and stride length
Miyai *et al*[41]	Body weight supported treadmill training	45-min session, 3 times/wk, for 4 wk	Decrease of symptoms (UPDRS scale) and improvements in gait speed
Miyai *et al*[37]	Body weight supported treadmill training	45-min session, 3 times/wk, for 4 wk with progressive increasing of belt speed	Improvement in gait speed, decreasing of steps number over a 10 m distance. The effects lasted over 1 and 4 mo of follow up
Herman *et al*[36]	Intensive treadmill protocol	30 min session, 4 times/wk, for 6 wk with progressive increasing of belt speed	Improvement in gait parameters (speed, swing time variability), balance, motor symptoms (UPDRS scale) and in quality of life
Cakit *et al*[21]	Speed dependent treadmill training	30 ± 5 min session, 8 wk	Improvement in tolerated speed and distance walked, in balance and reduction in fear of falls
Fisher *et al*[29]	Body weight supported treadmill training	3.0 metabolic equivalents session, 3 times/wk for 8 wk	Improvement in gait speed, step and stride length, hip and ankle joint excursion, and decrease cortico-motor excitability
Protas *et al*[39]	Walking in all 4 directions and step training	1 h session, 3 times/wk for 8 wk	Reduction of falls, improvement in gait speed and stride length, improvement in dynamic balance
Rose *et al*[42]	Skipping, sprinting, walking, running and jumping on a lower body positive-pressure antigravity treadmill + spatial cues	1 h session, 3 times/wk for 8 wk	Improvement in gait and functional capacity, better quality of life, improvement in motor symptoms (UPDRS scale)

UPDRS: Unified Parkinson's Disease Rating Scale.

ated the treadmill training protocol carried out over 8 wk. Cakit *et al*[21] trained their patients with a SDTT protocol and described improvements in tolerated speed and distance walked, improved balance and reduced fear of falls. Fisher *et al*[29] demonstrated that high-intensity BWSTT 3 times a week over an 8-wk period improved: gait speed, step and stride length, hip and ankle joint excursion, body weight distribution, and, as pointed by the authors, "importantly" in CSP length, which in fact translates into decreased corticomotor excitability. Protas *et al*[39] designed a specific treadmill protocol, in which patients walked in all 4 directions at a speed higher than normal gait; "step training" was also included, as the treadmill belt was suddenly switched off during the exercise. Results showed a substantial reduction in falls, increased gait speed and stride length as well as improved dynamic balance. In the latest study[42], different tasks were performed by patients, such as skipping, sprinting, walking, running, jumping, and also spatial cues where used, with the patient working on a lower body positive-pressure antigravity treadmill. The study by Rose *et al*[42] showed significant and promising results for this brand new, high intensity protocol: improvements affected the score for Movement Disorders Society- UPDR Scale, better quality of life (32% improvement at Parkinson's Disease Questionnaire-39), and increased gait and functional capacity [assessed with the 6-minute walk test (6MWT)].

Finally, it is mandatory to note that treadmill training may generate positive effects also on the central nervous system, namely, dopamine availability and corticomotor excitability, 2 central elements which are impaired by PD and cause motor disorders. Different interventions of treadmill training are shown in Table 2.

Cueing strategies

It is known that the normal footstep pattern is not lost in PD, rather there is a problem in activating the correct stepping response for a given context. Several studies demonstrated that the interaction between the basal ganglia and supplementary motor area (SMA) is disrupted during movement performance. The SMA normally prepares for a forthcoming predictable movement with a steady increase in neuronal activity during the premovement period. Once the external signal to move occurs, the neuronal activity in the SMA abruptly ceases. The basal ganglia discharge with brief bursts of phasic activity at the end of submovements performed in a sequence. This activity represents an internal cue which triggers the rapid drop in SMA neuronal activity. If the basal ganglia cue was absent or disturbed, as in PD, then it is possible that the SMA preparatory activity would be disturbed, leading to an abnormally executed movement[43].

As a consequence of the dopaminergic neuronal defection, the basal ganglia do not provide the correct cues for motor sequences to cortical motor areas (primary motor cortex, premotor cortex and supplementary motor area). This defect justifies the patient's inability to prepare and maintain the execution of complex and well-learned movements, *e.g.*, walking[9,22,34,43-45]. Specific gait impairments like FoG and festination are probably caused by an internal lack of rhythmic cues[9,14,46].

Conceivably, physical therapy should aim to compensate the physiologic defections at the base of the motor impairments[14,32,47]. In this setting, adapted exercise should be planned with the aim of reaching the cortical motor areas and bypassing the affected basal ganglia, in order to improve motor performance. External cues seem to be an effective method[14,27,28], and many authors demonstrated their efficacy. There are 3 types of cues which have been mainly studied: visual (transverse stripes placed on the walk path), auditory (music or simple beeps and sounds) and somatosensory (vibrations). Visual cues seems to facilitate Parkinsonian gait since they focus patient's concentration during the act of walking, thus making it a vol-

Table 3 Results of cueing training

Thaut *et al*[49]	Rhythmic auditory cues	Walking, stop-and-go, stair stepping listening to music	30 min/d for 3 wk	Improvement in gait speed, stride length and cadence
McIntosh *et al*[50]	Rhythmic auditory cues	Walking and stop-and-go listening to music	Single session	Improvement in gait speed, stride length and cadence
del Olmo *et al*[34]	Rhythmic auditory cues	Walking in different condition (with or without metronome cadence)	1 h/d, for 5 times/wk for 4 wk	Improvement in gait temporal stability
Azulay *et al*[48]	Dynamic and static visual cues	Walking on a 12-m walkway with parallel transversal white stripes with normal/ stroboscopic lights	Single session	Increased velocity and stride length in the normal lights condition, suggesting the role of a specific visuo-motor pathway elicited by the moving cues
Rochester *et al*[35]	Auditory + visual cues	Little itinerary performed at home	Single session	Auditory cues improved performance (stride length) in the functional task, and a tendency for increased walking speed was noticed with both types of cue
Nieuwboer *et al*[23]	Visual, auditory or somato-sensory cues	Home-based cueing training program	30 mine session, 3 d/wk for 3 wk	Improvement in posture, gait speed, step length, reduction of FoG episodes and increased confidence in gait tasks
Frazzitta *et al*[9]	Treadmill + external cues	Progressive treadmill training with auditory (musical beats) and visual cues	20 min every day for 4 wk	Improvement in UPDRSIII, 6MWT, gait speed, stride cycle, FoG questionnaire

FoG: Freezing of Gait; 6MWT: 6-Minute walk test; UPDRS: Unified Parkinson's Disease Rating Scale.

untary task[11,48]. Auditory cues provide an external rhythm that bypasses the affected basal ganglia, thus improving gait performance, timing and cadence[9,7,33,49]. No information is given for the putative mechanism of somatosensory cues efficacy but the explanation may rationally be very similar to the auditory cues.

McIntosh and colleagues studied the effect of rhythmic auditory cues on the gait of PD patients. Their first study[49] was a 3-wk home-based gait training program: patients trained 30 min/d, with walking, stop-and-go and stair stepping performed while patients listened to music in a headset (music tempo was progressively increased during the experiment). There were significant improvements regarding gait speed, stride length and cadence. In the second protocol[50], auditory cues again facilitated subjects' gait, with significant improvements in gait speed (patients were able to walk at a speed higher than their maximal one), stride length and cadence. del Olmo *et al*[34] also assessed the effect of auditory cues on PD gait. Fifteen patients underwent gait training (which also included manual dual-tasking) with rhythmic sounds, 1 h per day, 5 d a month: auditory cues increased gait temporal stability, especially in those patients with greater impairment at baseline (a discovery that gains even more significance given that the level of gait impairment is proportional to PD severity).

Azulay *et al*[48] analyzed the effect of both dynamic and static visual cues on PD gait. Participants in the study session walked on a 12-m walkway with parallel transversal white stripes (visual cue) spaced at 45 cm intervals; patients walked first with normal lights on, and then with stroboscopic lights (used to suppress the perception of movement of the stripes). While performance significantly decreased due to the absence of dynamic cues (stroboscopic lights), patients increased velocity and stride length in the normal lights, suggesting the role of a specific visual-motor pathway elicited by the moving cues.

Most of the studies available in the literature compared the different type of cues. Rochester *et al*[35] examined the effect of both visual and auditory cues on a gait dual task performed at home: patients simply had to stand up, go to their kitchen, put 2 cups on a tray, walk back, leave the tray on a near table and sit down again. Auditory cues improved performance in the functional task (stride length), and a tendency for increased walking speed was noticed with both kinds of cue. The most complete study on external cues for PD is the RESCUE trial designed by Nieuwboer *et al*[23]. One hundred and fifty-three patients participated in this home-based cueing training program, in which patients had to perform gait tasks while receiving visual, auditory or somatosensory cues; training sessions lasted 30 min, 3 d per week, for 3 wk. The program generated many significant improvements in: posture and gait, gait speed, step length, reduction of FoG episodes (assessed through the FoG Questionnaire), and increased confidence in gait tasks (assessed with the Falls Efficacy Scale); importantly, 67% of patients preferred auditory cues, while the remaining 33% favored somatosensory cues.

Frazzitta *et al*[9] designed an experimental study, in which 40 patients who suffered FoG were divided into 2 groups: the first underwent progressive treadmill training with auditory (musical beats) and visual cues, while the second group followed a traditional protocol combining visual and auditory cues. Improvements were found in both groups for all the measured parameters (UPDRS-Ⅲ, 6MWT, gait speed, stride cycle, FoG questionnaire), showing the positive effect of both cues on motor performance. Table 3 summarizes the cueing strategies.

Improving physical capacity: Resistance training

Due to both central and peripheral causes, PD patients suffer muscle weakness (sarcopenia). Weakness in the lower limbs particularly affects basic daily tasks such as

standing up from a chair and walking. Moreover, sarcopenia is considered a secondary cause of bradykinesia[51]. Muscle weakness is also strongly related to impaired balance, since it reduces the ability to respond to postural and balance modifications[51]. When considering this observation, its association with the risk of falls is straightforward. Moreover, the consequences of balance loss (falls, injuries, immobilization) may adversely contribute to the maintenance of bone mass density in the hip[52], increasing the risk of hip fracture.

In PD, the nigral dopaminergic deficit results in an increase in tonic inhibition of the thalamus and reduction in the excitatory drive to the motor cortex leading to disruption of cortical activation of the muscle. Conceivably, this disorder may result in impaired motor unit recruitment and could contribute both to bradykinesia and muscle weakness.

When considering these impairments, resistance training has been proposed as an efficient intervention aimed at reducing muscle weakness, bradykinesia, balance problems, as well as improving bone parameters, physical functioning, ADLs and the quality of life[52-54].

Several observations suggest that resistance training may facilitate functional plasticity in the cortex and muscle activation patterns. For this reason, in PD patients, this kind of training could be therapeutic to modify the activity in the cortex and basal ganglia, as well as the connectivity between and within these structures. Indeed, several studies showed that resistance training resulted in an increase in electromyographic activation, possibly explained by improved motor unit recruitment, increased firing rate, and better synchronization[54].

The first resistance training intervention for PD was evaluated by Scandalis *et al*[55] in 2001. Their protocol included exercises for quadriceps, hamstring, calves and abdominal muscles. Training sessions took place twice a week for 8 wk. The authors described improvements in strength, gait speed and stride length.

Several studies assessed the effects of progressive resistance exercise (PRE), which uses a high resistance load progressively increasing during the period of training. In the study of Schilling *et al*[56], patients performed resistance training for lower limb muscles twice a week for 8 wk: as expected, leg strength was significantly improved. Hirsch *et al*[57] evaluated the efficacy of PRE for balance parameters. Patients were assigned to 2 different groups: while group 2 simply performed balance exercises, group 1 trained balance plus high-intensity PRE for knee and ankle muscles. Sessions took place 3 times per week on non consecutive days for 10 wk. Both groups improved balance, strength and reduced falls, but the group treated with PRE and balance training performed better and their results were greater. PRE, as shown by the study of Hass *et al*[53], may also improve typical walking impairment of PD such as gait initiation. Their 10-wk PRE program, which focused mainly on lower limb muscles, generated improvements both in postural adjustment and spatiotemporal parameters during gait initiation, and consequently muscle strength. O'Brien *et al*[58] focused more on the patient's perception of a PRE protocol. After 20 sessions (performed twice weekly), the researchers interviewed the participants. Patients reported physical and psychological benefits from the PRE program and expressed positive feedback as well as the intention to attend future programs.

Another very efficient strengthening method is eccentric training. The advantage of this type of conditioning lies in the fact that eccentric contraction can generate high forces and perform more work (and thus increase strength quicker) while requiring less energy when compared with concentric contraction, thus reducing fatigue. Dibble and colleagues rightly speculated on this assumption, and after having assessed the safety and feasibility of high-intensity eccentric training for PD patients, they evaluated its benefits. The protocol was the same for their 2 studies[59,60]: 2 groups (experimental and control) underwent the same exercise program, which included calisthenics, treadmill, balance training and conditioning 3 times per week for 12 wk; the only difference regarded lower muscle conditioning, for which the experimental group performed high-intensity eccentric exercise, while the control group underwent traditional strength exercises. Convincingly, in both studies, the experimental group results showed significant improvement in all tested parameters, namely: muscle hypertrophy, strength, mobility, bradykinesia, quality of life and UPDRS score (Table 4).

Balance training

Degeneration of the basal ganglia involves several physiological systems essential for balance control. Dysfunction of the basal ganglia influences the ability of central nervous system to translate sensory information (somatosensory, visual and vestibular) into a single reference frame, which is important for assessment of limb and body position in relation to the environment. Deficient motor regulation in PD manifests as poor inter-segmental coordination, difficulties adopting postural synergies and delayed adjustment in motor commands when moving from one task to another. To be specific, balance training needs to target functions, or impairments, of balance control associated with PD symptoms[61].

As already discussed, postural instability is probably the symptom with the lower response to pharmacotherapy[10,11,24]. A reduced ability to adapt to balance changes and perturbations automatically increases the risk of falling as well as the consequent injuries such as hip fracture[11]. The need for an integrative therapy that would ameliorate the PD patient's response is evident. With this aim, exercise interventions focused specifically on balance have been developed and evaluated.

Tai Chi, a Chinese martial arts discipline, has been proposed as a useful exercise program for PD patients since it encompasses techniques such as weight shifting, slow and controlled movement, trunk rotations, different stances, multidirectional stepping, and maintenance of postures that directly target PD balance and gait[62,63]. Many authors evaluated the effect of a Tai Chi program in PD patients (Table 5).

Table 4 Effects of resistance training

Scandalis *et al*[55]	Exercises for quadriceps, hamstring, calves and also abdominal muscles	2 times/wk for 8 wk	Improved strength, gait speed and stride length
Schilling *et al*[56]	PRE for lower limbs muscles	2 times/wk for 8 wk	Improved leg strength
Hirsch *et al*[57]	Balance exercise plus high-intensity PRE for knee and ankle muscles	3 times/wk for 10 wk	Improved balance, strength and reduced falls
Hass *et al*[53]	PRE program, focused mainly on lower limbs muscles	2 times/wk for 10 wk	Improvement in both postural adjustment and spatiotemporal parameters during gait initiation (protective effect on falls), and improved muscle strength
O'Brien *et al*[58]	PRE	2 times/wk for 10 wk	Physical and psychological benefits
Dibble *et al*[59,60]	High Intensity eccentric training exercise program for lower muscles which included calisthenics, treadmill, balance training and conditioning	3 times/wk for 12 wk	Improvement in muscle hypertrophy, strength, mobility, bradykinesia, Quality of life and UPDRS score

PRE: Progressive resistance exercise; UPDRS: Unified Parkinson's Disease Rating Scale.

Table 5 Effects of balance training

Li *et al*[62]	Tai Chi *vs* resistance training and stretching	60 min sessions 2 times/wk for 24 wk	Tai Chi group improved their postural stability significantly more than both the other groups; stride length and velocity, strength, timed up-and-go test, functional reaching and UPDRS-Ⅲ score were significantly higher in the Tai Chi group when compared with stretching; Tai Chi improved stride length, reduced rate of falls at follow up and, as shown by the posturography, there was a reduction of deviations of movement, which the authors suggest to be a reduction of dyskinesia
Hackney *et al*[63]	Tai Chi program	60 min session for 10-13 wk (total 20 session)	Improved global and motor symptoms (UPDRS and UPDRS-Ⅲ), balance, tandem stance, one leg stance, backward walking, and gait endurance (6MWT)
Schmitz-Hubsch *et al*[24]	Qi Gong program	90 min weekly training for 2 mo	Intervention showed a "stabilizing effect on PD symptoms": specifically, postural instability improved, as well as UPDRS-Ⅲ score. Also, autonomic dysfunction (constipation and pain) decreased, and during physiotherapy sleep disturbances and daytime sleepiness diminished

6MWT: 6-Minute Walk Test; UPDRS: Unified Parkinson's Disease Rating Scale.

Li *et al*[62] compared the effect of a Tai Chi program with resistance training and stretching (used as control). Subjects in each group trained twice a week for 6 mo. The Tai Chi training program was particularly designed for balance and gait training. Results showed that the Tai Chi group improved their postural stability significantly more than either of the other groups. Stride length and velocity, strength, timed up-and-go test (which evaluates static and dynamic balance and gait), functional reaching and UPDRS-Ⅲ score were significantly higher in the Tai Chi group when compared to stretching. Furthermore, 2 findings are of particular significance: the rate of falls at follow-up was lower in Tai Chi group, and, as shown by posturography, there was a reduction in deviations of movement as a result of reduced dyskinesia. Hackney and Earhart[63] evaluated the effect of 20 lessons (over 13 wk) of Tai Chi. Patients improved their global and motor symptomatology (UPDRS and UPDRS-Ⅲ), balance, tandem stance, one leg stance, backward walking, and gait endurance (6MWT). In addition, "patients reported enjoyment in the protocol and physical and psychological improvements".

Schmitz-Hübsch *et al*[24] determined the effect of another Chinese exercise therapy, Qi Gong (which includes posture, breathing techniques and attention strategies). Their 2-mo intervention showed a "stabilizing effect on PD symptoms": specifically, postural instability improved, as well as UPDRS-Ⅲ score. Moreover, autonomic dysfunction (constipation and pain) decreased, and during physiotherapy sleep disturbances and daytime sleepiness diminished.

Finally, worthy of mention is the innovative, balance-specific program designed and assessed by Esculier *et al*[64]. The researchers submitted PD patients to a home-based program using Nintendo Wii Fit with balance board. The device focuses on balance tasks and visual feedback of movements is constantly provided, together with auditory and proprioceptive cues. Additionally, the console and the games seemed to be very enjoyable and motivating for the patient. In this study, patients trained for 40 min, 3 d a week, for 6 wk. Results were meaningful, with improvements in static and dynamic balance, gait, functional strength of the lower limbs, one-leg stance time and reduced fear of falling.

Dance

Due to its nature, dance appears to be one of the most effective exercise protocols for PD patients. Indeed, as discussed by Dr. Earhart, all the recommended key areas[28] for physical therapy in PD are met[65]. Music serves as an

Table 6 Results of dance training

Hackney *et al*[66,69]	Tango	60 min session, 2 d/wk for 10 wk (total 20 sessions)	Decreased UPDRS score, improved balance, reduced fear of falling. Trends of improvement for FoG and at Timed Up and Go test
Hackney *et al*[67]	Tango *vs* Foxtrot	60 min session, 2 d/wk for 13 wk (total 20 session)	Both types of dance improved gait speed, balance, backward stride length, cardiovascular function and symptoms (UPDRS); only Tango generated improvements for FoG
Duncan *et al*[70]	Tango in patients "off medication"	60 min session, 2 d/wk for 12 mo	Bradykinesia and motor symptoms severity (assessed with MDS-UPDRS-Ⅲ) were reduced; gait speed, balance, dual task walking speed and upper extremity function all improved; rigidity, FoG and gait endurance remained stable, but in the control group they progressively worsened, ("braking" effect on PD progression)

PD: Parkinson's disease; UPDRS: Unified Parkinson's Disease Rating Scale.

external cue, thus facilitating motor performance; specific movement strategies are taught; balance is trained, especially in its dynamic form. Although not directly addressed, dance may improve strength and flexibility. Finally, when trained at the right intensity, dance promotes cardiovascular functioning as an optimal form of aerobic exercise. This discipline may be considered an ideal choice among all adapted physiotherapy programs, since it addresses specific Parkinsonian impairments such as walking backward, turning and multitasking. Furthermore, the Tango appears to be the most Parkinson-specific discipline, since the basic step used is walking; frequent stops and starts are common (thus challenging the patient's start hesitation); directional changes and turning are included, and dancing at different rhythms and speeds addresses bradykinesia. Furthermore, some techniques like stepping or tapping the partner's feet, crossing feet, and shifting the body weight from one leg to another, are very similar to strategies used in rehabilitation of FoG[65-68].

Researchers have evaluated 2 dance discipline so far: waltz/foxtrot and, of course, Tango. In 2 studies, Hackney *et al*[66,67] reported that 20 Tango sessions diminished symptoms, improved balance, and reduced fear of falling. Moreover, trends of improvement in FoG and the Timed Up and Go test (which measures static and dynamic balance) were found. Significantly, in both studies, half of the patients decided to participate in additional Tango sessions; an unequivocal sign of the patients' enjoyment of the protocol. Hackney and Earhart[68,69] also tried to evaluate the difference between partnered and non-partnered dance, since "the partner's importance and influence remains equivocal". Again, 20 Tango sessions generated improvements in gait, balance and functional mobility. No difference were found between the 2 groups, but the authors suggested that, for safety reasons, a partner may be useful for patients in the later stages of PD since the partner acts as a balance support. Duncan and Earhart[70] reported a 12-mo study, in which 52 patients assigned to a Tango group trained twice a week for 1 h. In this study, patients were tested only off medication "to ultimately determine whether exercise may be disease modifying". Significantly, patients benefitted greatly from the long-term protocol: motor symptom severity (assessed with MDS-UPDRS-Ⅲ) was reduced, as well as bradykinesia. Gait speed, balance, dual task walking speed and upper extremity function all improved. Rigidity, FoG and gait endurance remained stable, but in the control group they progressively worsened. These results confirm how exercise can have a "braking" effect on PD progression (Table 6).

CONCLUSION

With the exception of tremors, tailored physical activity has shown to improve all the prominent motor symptoms of PD patients including those harmful disturbances such as FoG, stride variability and balance impairments. Furthermore, it has been clearly demonstrated that each of the different types of physical activity resulted in a better quality of life. It is therefore reasonable to state that tailored physical activity could be considered as a valid intervention to be included in the therapeutic program of PD patients.

Each training protocol has specific technical characteristics targeting different PD deficiencies. Studies applying treadmill training described improvements in patient gait in quick FoG, festinating gait and balance loss. Moreover, in several studies researchers reported improvements in UPDRS, functional capacity and quality of life.

The use of external cues to bypass the affected basal ganglia also showed promising results. Most of the spatiotemporal walking parameters, such as gait speed, stride variability, cadence, and step length improved after the use of external cues. Nieuwboer *et al*[23] also reported that external cues reduced FoG events and improved patient confidence when considering the risk of falls.

Dancing is an alternative program which seems promising and efficient for the treatment of PD symptoms. Specific Parkinsonian patterns such as bradykinesia, dynamic balance, backward walking, turning and multitasking are directly targeted with dance. In addition, the Argentinian Tango can be labeled as a "Parkinson-specific discipline". The effect of the dance relies on its social and enjoyable nature, which stimulates patient compliance for longer periods, thus potentially enhancing the positive effects of the program and expanding its beneficial effect to the emotional and psychological sphere.

The beneficial effects of resistance training are not limited to muscle hypertrophy and improved strength. Indeed, significant improvements have been described when considering balance, bradykinesia, gait, mobility

and quality of life. Balance-specific protocols, mainly Tai Chi, resulted in significant improvements when considering gait, balance and posture, finally leading to a reduced risk of falls.

As discussed by most of the authors cited, the main limit of adapted physical therapy in PD patients relies in the lack of a standardized therapeutic protocol for common use. Even if future investigations should address this issue, we strongly support the adoption of an individualized approach. Indeed, PD is a very complex disease, with different and fluctuating symptoms which affect the patients almost uniquely. Consequently, a physical therapy protocol should not be standardized, but tailored and individualized to the patient's personal condition in order to target his/her precise motor impairments.

Consequently, it seems crucial to educate the patient early about the benefit of an active lifestyle, including regular participation in an specific physiotherapy program, in order to promote independence, physical functionality and quality of life. Patients should choose an enjoyable program in order to promote adherence. Based on the available experience, training sessions should last 60-75 min at least 3 times per week, especially in the earlier stages. It seems to be useful to perform stretching exercises daily in order to reduce rigidity and improve joint and muscular capability.

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REVIEW

Closer look at white-coat hypertension

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Abstract

This review aims to clarify novel concepts regarding the clinical and laboratory aspects of white-coat hypertension (WCHT). Recent studies on the clinical and biological implications of WCHT were compared with existing knowledge. Studies were included if the WCHT patients were defined according to the 2013 European Society of Hypertension guidelines, *i.e.*, an office blood pressure (BP) of $\geqslant$ 140/90 mmHg, a home BP of $\leqslant$ 135/85 mmHg, and a mean 24-h ambulatory BP of $\leqslant$ 130/80 mmHg. WCHT studies published since 2000 were selected, although a few studies performed before 2000 were used for comparative purposes. True WCHT was defined as normal ABPM and home BP readings, and partial WCHT was defined as an abnormality in one of these two readings. The reported prevalence of WCHT was 15%-45%. The incidence of WCHT tended to be higher in females and in non-smokers. Compared with normotensive (NT) patients, WCHT was associated with a higher left ventricular mass index, higher lipid levels, impaired fasting glucose, and decreased arterial compliance. The circadian rhythm in WCHT patients was more variable than in NT patient's, with a higher pulse pressure and non-dipping characteristics. Compared with sustained hypertension patients, WCHT patients have a better 10-year prognosis; compared with NT patients, WCHT patients have a similar stroke risk, but receive more frequent drug treatment. There are conflicting results regarding WCHT and markers of endothelial damage, oxidative stress and inflammation, and the data imply that WCHT patients may have a worse prognosis. Nitric oxide levels are lower, and oxidative stress parameters are higher in WCHT patients than in NT patients, whereas the antioxidant capacity is lower in WCHT patients than in NT patients. Clinicians should be aware of the risk factors associated with WCHT and patients should be closely monitored especially to identify target organ damage and metabolic syndrome.

Key words: White-coat hypertension; Ambulatory blood pressure; Target organ damage; Glucose dysregulation

Core tip: There is contradictory information regarding the clinical presentations and prognosis of white-coat hypertension (WCHT). This review aims to summarize recent research and compare it to existing knowledge about WCHT. Published studies on the prevalence of WCHT, the associated target organ damage and cardiovascular markers, and WCHT patient prognosis were reviewed. WCHT may be a marker of future obesity and metabolic syndrome, is related to glucose dysregulation and left ventricular hypertrophy and may progress to sustained hypertension. Clinicians should be aware of the risk factors associated with WCHT, and patients should be closely monitored, especially to identify target organ damage and metabolic syndrome.

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DEFINITION OF WHITE-COAT HYPERTENSION

According to the 2013 guidelines published by the Euro-

pean Society of Hypertension and the European Society of Cardiology for the management of hypertension white-coat hypertension (WCHT) is defined for patients who are not taking medication as an office blood pressure (BP) of ⩾ 140/90 on at least three occasions in the presence of a health care worker (particularly a physician),with normal 24-h (⩽ 125-130/80 mmHg) and day ambulatory BP monitoring (ABPM) (⩽ 130-135/85 mmHg) or a normal home BP (average of several readings, ⩽ 130-135/85 mmHg)[1]. There were no changes in the definition of WCHT in the JNC8[2]. The American Heart Association (AHA) recommends home BP monitoring for patients with pre-hypertension (120-139/80-89 mmHg) and for patients diagnosed with hypertension (systolic BP ⩾ 140 mmHg or diastolic BP ⩾ 90 mmHg)[3].

It is recommended that at least two 24-h ABPM measurements be recorded to confirm WCHT[4]. WCHT can be divided into two subgroups: partial WCHT, where either the ABPM or the home BP readings are elevated; and true WCHT, in which both the ambulatory and home BP values are normal[5].

WCHT should be differentiated from the white coat effect, which is a rise in BP in response to the presence of a medical practitioner and may be observed in all types of patients, from normotensive (NT) persons to patients with sustained hypertension (SHT) in the presence or absence of therapy[6].

The prevalence of WCHT, its associations with other clinical conditions and vascular biomarkers, the risk of target organ damage and patient prognosis in comparison with other forms of hypertension will be discussed in this review.

Studies were included if the WCHT patients were defined according to the 2013 European Society of Hypertension guidelines. WCHT studies published since 2000 were selected, although a few studies performed before 2000 were used for comparative purposes.

THE PREVALENCE OF WHITE-COAT HYPERTENSION

The Finn-Home study was performed on 1540 untreated participants with an age range of 44 to 75 years. Two hundred thirty-three patients had WCHT. The prevalence of WCHT was 15.1%. The home BP levels of the WCHT patients were higher than those of the NT individuals. The WCHT group was older and had a higher proportion of men; WCHT patients had more metabolic risk factors than NT individuals[7].

The PAMELA study was a population study performed in Monza, Italy. BP was measured in the office and, twice daily, by subjects at home. The investigators assessed the cardiovascular and all-cause mortality over 16 years (1992-2008) in 2051 patients with an age range of 25-74 years. The prevalence of WCHT among untreated hypertensive patients was estimated at 15%-45%, and WCHT was associated with non-smoking, female gender and increasing age. The prevalence of partial WCHT was 58%, whereas the prevalence of true WCHT was 42%[5].

Pickering *et al*[8] reported that the prevalence of WCHT was 21% in a 1988 study of 292 patients. WCHT patients tended to be female, be younger, and have a lower body weight.

PREVALENCE OF WHITE-COAT HYPERTENSION IN PATIENTS WITH CHRONIC RENAL DISEASE

The prevalence of WCHT was 15% among 355 long-term hemodialysis patients. When a pre-dialysis BP threshold of 140/90 mmHg was used to classify patients into BP categories, the prevalence of WCHT was 26%[9].

In a study by Bangash *et al*[10], the prevalence of WCHT was 18.3% in 980 patients with chronic renal disease (CRD). The threshold for identifying hypertension based on clinic and ambulatory BP measurements strongly influenced the risk of being diagnosed with masked hypertension (MHT) rather than WCHT. In studies of CRD patients with overt proteinuria, the lower threshold for WCHT (< 140/90 mmHg) was responsible for the increased prevalence of WCHT.

PREVALENCE OF WHITE-COAT HYPERTENSION IN TYPE 2 DIABETES MELLITUS PATIENTS

In a Chinese study of 473 patients, the prevalence of WCHT was 7.36% in the overall population, 6.13% in male patients and 8.88% in female patients ($P < 0.05$). Age, etiology of type 2 diabetes mellitus (DM) and male gender were dependent factors, whereas female gender, smoking and alcohol consumption were independent risk factors for WCHT in patients with type 2 DM[11]. These findings are in accordance with previous studies, and the characteristics of WCHT in diabetic patients are similar to those in the general population.

WHITE-COAT HYPERTENSION AND METABOLIC SYNDROME

According to the 2009 Joint Statement on Metabolic Syndrome, metabolic syndrome is defined as the presence of abnormalities in three of the following five characteristics: waist circumference, triglyceride levels, high-density lipoprotein levels, BP and fasting blood glucose[12].

Helvaci *et al*[13,14] analyzed 955 patients (566 females and 389 males) and suggested that WCHT is not a predisposing factor for hypertension (HT) or atherosclerosis but rather it is an alarm signal. There was an increasing prevalence of obesity, impaired glucose tolerance (IGT) or DM, and coronary heart disease (CHD) between the WCHT and HT groups compared with the NT group. According to this study, the prevalence of dyslipidemia

was the highest in the WCHT group (41.6%, $P < 0.05$), followed by 35.5% in the SHT group and 19.6% in the NTgroup.

In another study by Björklund *et al*[15], 602 male patients aged 50 years and older who had WCHT were followed for 20 years. Their baseline body mass index (BMI) values were similar to those of the individuals in the NT group (23.0 kg/m^2 *vs* 23.8 kg/m^2) at age 70. However, metabolic abnormalities (insulin sensitivity, elevated blood glucose, and increased serum insulin) and elevated heart rate (HR) developed over time in patients with WCHT and SHT. A lower BMI and a more favorable dietary fat composition predicted the development of WCHT as opposed to SHT.

The PAMELA study demonstrated that patients with WCHT had similar baseline BMI values to patients with SHT(27 ± 4.3 kg/m^2 *vs* 27.4 ± 5.2 kg/m^2) but had higher fasting glucose levels compared with NT individuals (93.2 ± 20.9 mg/dL *vs* 85.5 ± 12.5 mg/dL, $P < 0.05$). At the end of the 10-year follow-up period, patients in the WCHT group were more likely to develop diabetes than those in the NT group (OR = 2.9)[16].

A study conducted in Turkey by Afsar[17] revealed that the progression from sustained NT to SHT involves an increase in serum uric acid levels, impaired fasting glucose levels, and increases in BMI and waist circumference. The presence of metabolic syndrome was highest in people with SHT and lowest in those with sustained NT ($P < 0.0001$). This study concluded that the changes in these parameters were not as substantial in the WCHT group as they were when comparing the MHT and SHT groups to the NT group.

Several other studies have shown that the prevalence of impaired fasting glucose levels and abnormal glucose tolerance test results is higher in WCHT patients than in NT individuals. These findings suggest that WCHT is associated with glucose dysregulation and an increased risk for diabetes[18,19].

Therefore, WCHT is an initial sign of deteriorating health. It commonly accompanies hyperlipidemia, elevated fasting glucose levels and a tendency toward being overweight.

WHITE-COAT HYPERTENSION IN ELDERLY PATIENTS

Two studies have analyzed WCHT in elderly people. In the first study, which was published by Hekman *et al*[20], elderly women (age range, 60-83 years; mean age, 69 ± 7 years) with WCHT had a higher SBP than NT elderly women between the hours of 8 am and 12 pm (133 ± 8.0 mmHg *vs* 123 ± 9.0 mmHg, $P < 0.001$). The BP variability was higher in the WCHT group only during the wakeful period (between 7 am and 11 pm, $P = 0.02$).

Age and BMI positively correlated with mean SBP at night. In the elderly women with WCHT, a higher SBP was associated with increasing age and BMI ($P = 0.015$ and $P = 0.055$, respectively). Elderly women with WCHT were more likely to smoke ($P = 0.014$) and snore ($P = 0.046$)[20].

In the second WCHT study on the elderly, Franklin SS. and colleagues analyzed 1168 untreated subjects with a mean age of 48.8 ± 16.6 years and with isolated systolic hypertension (ISH); 28.6% of the study participants had WCHT. The cardiovascular risk in untreated WCHT patients with ISH was similar to that in NT individuals ($P = 0.63$). Compared with the untreated NT individuals, individuals with WCHT undergoing treatment for ISH as well as treated NT individuals were at a higher cardiovascular risk ($P < 0.01$)[21]. The results suggested that age, BMI and the need for treatment increased the cardiovascular risk in elderly patients with WCHT.

CIRCADIAN RHYTHMS IN PATIENTS WITH WHITE-COAT HYPERTENSION

BP, HR, cardiac output and serum catecholamine levels increase during the day and decrease during the night. These changes enable an organism to adapt to the need for higher activity levels while awake. The decrease in BP at night is defined as "dipping," and patients who fail to show this pattern are called "non-dippers"[22].

A study by Koroboki *et al*[22] determined that WCHT patients have the same circadian pattern as NT, MHT and untreated HT patients; however, the daytime and nighttime pulse pressures were higher in WCHT patients than in NT individuals, with nighttime pulse pressures reaching those in MHT patients. Circadian BP and HR profiles in MHT and WCHT patients have been compared with those in NT patients and in treated and untreated SHT patients using ambulatory BP measurements.

Pierdomenico *et al*[23] studied 12 NT, 12 WCHT and 12 SHT patients in 2000. The subjects underwent ABPM. Power spectral analyses of the R-R intervals were performed to obtain the low and high frequency components, concomitant with 24-h urine testing for epinephrine and norepinephrine. This study demonstrated that patients with WCHT and SHT have similar circadian patterns based on ABPM; however, the other findings indicated sympathetic overactivity throughout the day in SHT patients but not in WCHT patients, suggesting that the two conditions may have different pathophysiological backgrounds[23].

Vyssoulis *et al*[24] classified WCHT patients according to the presence of accompanying metabolic syndrome traits and thus divided the patients into two groups based on the presence ($n = 522$) or absence ($n = 1778$) of metabolic syndrome. Patients with WCHT and a greater number of metabolic syndrome traits had non-dipping characteristics, along with elevated nighttime SBP levels that are indicative of an increased cardiovascular risk[24].

WHITE-COAT HYPERTENSION AND TARGET ORGAN DAMAGE

In 2003, Karter *et al*[25] studied 50 NT, 90 WCHT and

101 SHT subjects and reported that WCHT patients had a higher BMI and a greater left ventricular mass index (LVMI) than NT individuals ($P < 0.001$). Urinary albumin excretion was similar in WCHT patients and in those with SHT. No difference in renal function between WCHT and HT patients was noted by Pierdomenico *et al*[26].

In the PAMELA study, Cardillo, Weber, Mancia and Mulè reported that LVMI was increased in WCHT patients compared with NT patients ($P < 0.01$), whereas Pierdomenico and Hoeghelm found no difference in LVMI[5,26-31].

Arterial compliance was lower in WCHT patients than in the NT group in studies by Karter's ($P < 0.001$) and Gomez[25,32]. Turfaner *et al*[33] analyzed 47 dipper and 43 non-dipper WCHT patients and determined, that non-dipping in WCHT patients was related to decreased arterial compliance and that the global risk load for target organ damage was higher in non-dipper WCHT subjects.

The carotid artery intimal media thickness (IMT), which is used to measure the progression of atherosclerosis in WCHT patients, has been reported to be similar in NT and WCHT individuals (see studies by Pierdomenico, Karter, Roman and Gariepy[25,34-36]).

In contrast, the HARVEST study by Puato *et al*[37] compared the baseline and follow-up IMT values in 35 WCHT, 20 NT and 39 SHT patients over five years. The baseline ($P = 0.004$) and follow-up ($P < 0.01$) IMT values were significantly higher and increased faster in WCHT patients than in NT controls. There was no significant difference between patients with WCHT and those with SHT ($P = 0.27$). This increase in IMT was associated with triglyceride levels, age and mean arterial pressure at ABPM, as determined by multivariate regression analysis[37].

In a cross-sectional survey that included 2915 Japanese patients aged ⩾ 40 years, the carotid IMT was significantly thicker in WCHT patients than in NT patients (0.73 mm *vs* 0.67 mm, $P = 0.001$)[38].

WHITE-COAT HYPERTENSION AND BIOVASCULAR MARKERS

Studies on endothelial damage and angiogenesis, which indicate an increased risk for a poor prognosis in WCHT patients, have suggested that WCHT is associated with significantly higher endothelin-1 and vascular endothelial growth factor levels[39].

There is controversy regarding the amount of Nitric Oxide (NO) in WCHT patients. Karter observed higher NO levels in WCHT patients compared with NT patients ($P < 0.001$). Karter *et al*[39], Pierdomenico *et al*[40] and Güven *et al*[41] showed no significant differences in NO levels in WCHT and NT patients. Pierdomenico *et al*[40] and Guven *et al*[41] demonstrated that NO levels were higher in WCHT patients than in SHT patients ($P < 0.05$). The difference was more significant in the Karter study ($P < 0.001$). In the Karter study, the threshold for clinical WCHT was defined as a DBP > 85 mmHg; in other studies, the threshold was defined as a BP ⩾ 140/90 mmHg, which may result in a difference in endothelial dysfunction parameters.

Homocysteine and asymmetric dimethylarginine levels were examined in two different studies with contradictory results. Pierdomenico found no significant differences in homocysteine levels among WCHT patients and NT patients, whereas Curgunlu *et al*[42,43] showed significantly higher homocysteine levels in WCHT patients ($P < 0.001$). Homocysteine levels were significantly higher in SHT patients than in WCHT patients, with P values of 0.0003 and $P < 0.001$ in the studies performed by Pierdomenico *et al*[44] and Curgunlu *et al*[42,43], respectively. Regarding inflammation, only one of three WCHT studies on C-reactive protein found a significant difference between the WCHT and NT groups[41,45,46].

The reports on oxidative stress markers in WCHT are conflicting. Among the oxidative stress parameters, paraoxonase (PON-1) levels were significantly lower ($P < 0.001$) and malondialdehyde (MDA) levels were higher ($P < 0.026$) in WCHT patients compared with NT patients, whereas ox-LDL was not significantly different between the NT, WCHT and SHT groups[47].

A study by Caner *et al*[48] on other oxidative stress parameters, such as protein carbonyl(PCO) and antioxidant capacity, showed that PCO was higher ($P < 0.001$) and that antioxidant markers(plasma thiol, plasma CuZn-SOD and erythrocyte glutathione) were lower ($P < 0.01$)in WCHT patients compared with the NT group. Plasminogen activator 1(PAI-1) and von Willebrand factor levels were not different between the WCHT and NT groups[45].

PROGNOSIS AND STROKE RISK IN PATIENTS WITH WHITE-COAT HYPERTENSION

Verdecchia *et al*[49] reported that the cumulative hazard for stroke in WCHT patients (based on ABPM) tended to increase after 6 years of follow-up and exceeded that of ambulatory hypertensive patients after 9 years of follow-up.

In Japan, 1332 subjects (872 females and 460 males, age ⩾ 40 years) who were representative of the Japanese population were followed for 10 years to monitor stroke risk as part of the OHASAMA study. There was no significant difference in outcome between WCHT and NT patients (daytime BP < 135/85 mmHg based on ambulatory BP)[50].

Pierdomenico *et al*[51] compared the cardiac and cerebrovascular risks in SHT and WCHT individuals, which were reported to be RR = 4.16, 95%CI: 1.48-11.6, $P = 0.007$, and RR = 4.12, 95%CI: 1.62-10.5, $P = 0.003$, respectively. There was no significant difference between the NT and WCHT individuals in this study, which followed 1732 subjects (1333 SHT, 399 WCHT, and 305 NT) for 6 years.

In another study, Pierdomenico *et al*[52] compared the cardiovascular risk in NT and WCHT patients and found no statistically significant differences, regardless of the

NT population type or the follow-up duration. They noted that the WCHT patients were more likely to be receiving drug treatment when compared with the NT patients.

The PAMELA study reported that in partial WCHT patients with either ABPM or home BP monitoring abnormalities, the incidence of fatal events was markedly increased, with a 60% higher fully adjusted risk of cardiovascular and all-cause mortality compared with NT controls; however, the risks of cardiovascular and all-cause mortality were not significantly different from those in NT subjects with true WCHT[5,29].

TREATMENT

Subjects with WCHT frequently have dysmetabolic risk factors and asymptomatic organ damage, which increase the cardiovascular risk. In these higher-risk individuals with WCHT, drug treatment may be considered in addition to appropriate lifestyle changes. Both lifestyle changes and drug treatment may also be considered when normal ambulatory BP values are accompanied by abnormal home BP values (or vice versa) because this condition is also characterized by increased cardiovascular risk. In the absence of additional cardiovascular risk factors, intervention may be limited to lifestyle changes but should include meticulous follow-up (including periodic out-of-office BP monitoring) because the out-of-office BP is often higher in WCHT patients than in truly NT individuals, and people with WCHT have a greater risk of developing organ damage or progressing to diabetes and SHT[1,2].

CONCLUSION

WCHT is a sign of deteriorating health. It is often accompanied by hyperlipidemia, elevated fasting glucose levels and a tendency toward being overweight. Based on the clinical and laboratory features, WCHT can be placed on a spectrum of BP disorders that extends between NT and SHT. Commonly, WCHT progresses to SHT, obesity and metabolic syndrome. In the elderly, the cardiovascular risk associated with WCHT increases with age, BMI and the need for treatment[53].

Patients with WCHT should be assessed for the presence of target organ damage and for the development of cardiovascular risk factors. These assessments should include an oral glucose tolerance test. Patients should be educated regarding increased cardiovascular and diabetes risks, with a special emphasis on maintaining or losing weight. Patients should limit salt intake and should not consume processed food. Patients with WCHT should be monitored for conversion to SHT (ABPM every six months or yearly and/or regular home monitoring).

In the future, with the increased use of ABPM or home monitoring, patients with WCHT will be identified more often and more easily; it may become possible to protect these patients from developing target organ damage[54].

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REVIEW

Dental movement acceleration: Literature review by an alternative scientific evidence method

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Abstract

The aim of this study was to analyze the majority of publications using effective methods to speed up orthodontic treatment and determine which publications carry high evidence-based value. The literature published in Pubmed from 1984 to 2013 was reviewed, in addition to well-known reports that were not classified under this database. To facilitate evidence-based decision making, guidelines such as the Consolidation Standards of Reporting Trials, Preferred Reporting items for systematic Reviews and Meta-analyses, and Transparent Reporting of Evaluations with Non-randomized Designs check list were used. The studies were initially divided into three groups: local application of cell mediators, physical stimuli, and techniques that took advantage of the regional acceleration phenomena. The articles were classified according to their level of evidence using an alternative method for orthodontic scientific article classification. 1a: Systematic Reviews (SR) of randomized clinical trials (RCTs), 1b: Individual RCT, 2a: SR of cohort studies, 2b: Individual cohort study, controlled clinical trials and low quality RCT, 3a: SR of case-control studies, 3b: Individual case-control study, low quality cohort study and short time following split mouth designs. 4: Case-series, low quality case-control study and non-systematic review, and 5: Expert opinion. The highest level of evidence for each group was: (1) local application of cell mediators: the highest level of evidence corresponds to a 3B level in Prostaglandins and Vitamin D; (2) physical stimuli: vibratory forces and low level laser irradiation have evidence level 2b, Electrical current is classified as 3b evidence-based level, Pulsed Electromagnetic Field is placed on the 4th level on the evidence scale; and (3) regional acceleration phenomena related techniques: for corticotomy the majority of the reports belong to level 4. Piezocision, dentoalveolar distraction, alveocentesis, monocortical tooth dislocation and ligament distraction technique, only had case series or single report cases (4th level of evidence). Surgery first and periodontal distraction have 1 study at level 2b and corticision one report at level 5. Multiple orthodontic acceleration reports on humans were identified by an alternative evidence level scale, which is a simple and accurate way of determining which techniques are better and have a higher rate of effectiveness. The highest level of evidence for a specific procedure to accelerate orthodontic dental movement up to October 2013 was surgery first followed by low level laser application, corticotomy and periodontal distraction located on level 2, recommendation grade b from this proposed scientific evidence-based scale.

Key words: Orthodontic movement; Evidence-based dentistry; Dental movement acceleration

Core tip: Orthodontic systematic reviews of randomized clinical trials, meta analysis and meta analysis network are difficult to develop due to a lack of high quality randomized clinical trials related to orthodontic therapies. The correct classification of the scientific literature fol-

lowing the evidence-based hierarchy facilitates the answers to specific clinical questions, and thus its application in every scientific subject. The resources available to speed up orthodontic movement had been widely examined. Due to a lack of evidence-based strength, the latter method cannot be taken into account in clinical protocols, thus we are left with the main already clinically proven methods: local injection of cellular mediators, physical stimuli, and surgically assisted orthodontics.

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INTRODUCTION

Any clinical inquiries in orthodontics should only be responded to after a thorough and critical analysis of the available scientific literature on the subject in question. Orthodontic patients deserve the highest level of care that is only possible through the strict use of the best available current information[1].

The best method for optimal information analysis involves stratified levels of evidence and grades of recommendations, regardless of the current classification. Evidence Based Dentistry is the conscientious, explicit, and judicious use of current best evidence in making decisions about the care of each patient[2-5].

The Oxford University evidence-based classification system includes every study carried out in humans using a very complete system (Table 1). The OCEBM levels have the advantage of offering levels of evidence for therapy, prognosis, diagnosis, differential diagnosis, and economic analysis all in a single table. In 2011, this system was simplified and modified, but left out the inclusion or possible adaptation of orthodontic scientific studies (Table 2).

In Table 3 an alternative scientific method to classificate scientific articles related to orthodontic therapies is proposed.

Orthodontic systematic reviews of randomized clinical trials (RCTs), meta-analysis and meta-analysis network are difficult to develop due to a lack of high quality randomized clinical trials related to orthodontic therapies. In the future, orthodontics ideally should develop and include prospective meta-analyses, thus avoiding the classic limitations of previous randomized clinical trials. The correct classification of the scientific literature following the evidence-based hierarchy facilitates the answers to specific clinical questions, and thus its application in every scientific subject.

The resources available to speed up orthodontic movement have been widely investigated in humans and animals. Due to a lack of evidence-based strength the latter cannot be taken into account in clinical protocols, thus we are left with the main already clinically proven methods: local injection of cellular mediators, physical stimuli, and surgically assisted orthodontics.

The main objective of this literature review was to analyze successful publications and the methods used to speed up orthodontic treatment and determine which publications carry a high evidence-based value.

LITERATURE SEARCH

The following clinical question was asked: Is there a way to move a tooth faster than conventional orthodontics? In order to begin the related literature search, the available methods to accelerate dental movement in adults were researched to determine which of these methods showed the highest level of scientific evidence.

Literature published in Pubmed from 1984 to October 2013 was reviewed, in addition to well-known reports that were not classified under this database.

To facilitate evidence-based decision making, guidelines such as the Consolidation Standards of Reporting Trials, Preferred Reporting Items for Systematic Reviews and Meta-analyses, and Transparent Reporting of Evaluations with Non-randomized Designs check list were used[6-8].

INCLUSION CRITERIA

Studies in any language and controlled or randomized clinical studies in humans.

EXCLUSION CRITERIA

In vitro or animal studies, reports that included non-effective methods to speed up dental movement and reports on the acceleration of dental movement that did not evaluate time in their research.

The studies were initially divided into three groups: local application of cell mediators, physical stimuli, and techniques that took advantage of the regional acceleration phenomena.

The articles were classified according to their level of evidence as shown in Table 3.

LOCAL APPLICATION OF CELL MEDIATORS

Local application of prostaglandins

The highest level of evidence corresponds to a 3B level from 3 publications: Yamasaki *et al*[9] developed a study which was divided into three phases. The first phase was on premolars which were to be extracted, on one side, they used sub mucosal injections of prostaglandin E1 (PGE1) and on the other side a vehicle substance was injected. The rate of movement of the teeth towards the buccal area was approximately 2-fold at the site of PGE1 injection. A similar result was obtained in the second phase where PGE1 injections were administered in the canine retraction areas for a period of 3 wk. The third

Table 1 The Oxford University evidence based classification applies and includes all studies performed on humans using a very complete system

Level	Therapy/ prevention, aetiology/harm	Prognosis	Diagnosis	Differential diagnosis/ symptom prevalence study	Economic and decision analyses
1a	SR (with homogeneity) of RCTs	SR (with homogeneity) of inception cohort studies; CDR" validated in different populations	SR (with homogeneity) of Level 1 diagnostic studies; CDR" with 1b studies from different clinical centres	SR (with homogeneity) of prospective cohort studies	SR (with homogeneity) of Level 1 economic studies
1b	Individual RCT (with narrow confidence interval")	Individual inception cohort study with > 80% follow-up; CDR" validated in a single population	Validating cohort study with good reference standards; or CDR" tested within one clinical centre	Prospective cohort study with good follow-up	Analysis based on clinically sensible costs or alternatives; systematic review(s) of the evidence; and including multi-way sensitivity analyses
1c	All or none	All or none case-series	Absolute SpPins and SnNouts	All or none case-series	Absolute better-value or worse-value analyses
2a	SR (with homogeneity) of cohort studies	SR (with homogeneity) of either retrospective cohort studies or untreated control groups in RCTs	SR (with homogeneity) of Level > 2 diagnostic studies	SR (with homogeneity) of 2b and better studies	SR (with homogeneity) of level > 2 economic studies
2b	Individual cohort study (including low quality RCT; *e.g.*, < 80% follow-up)	Retrospective cohort study or follow-up of untreated control patients in an RCT; Derivation of CDR" or validated on split-sample only	Exploratory cohort study with good reference standards; CDR" after derivation, or validated only on split-sample or databases	Retrospective cohort study, or poor follow-up	Analysis based on clinically sensible costs or alternatives; limited review(s) of the evidence, or single studies; and including multi-way sensitivity analyses
2c	"Outcomes" Research; ecological studies	"Outcomes" Research		Ecological studies	Audit or outcomes research
3a	SR (with homogeneity) of case-control studies		SR (with homogeneity) of 3b and better studies	SR (with homogeneity) of 3b and better studies	SR (with homogeneity) of 3b and better studies
3b	Individual case-control study		Non-consecutive study; or without consistently applied reference standards	Non-consecutive cohort study, or very limited population	Analysis based on limited alternatives or costs, poor quality estimates of data, but including sensitivity analyses incorporating clinically sensible variations
4	Case-series (and poor quality cohort and case-control studies)	Case-series (and poor quality prognostic cohort studies)	Case-control study, poor or non-independent reference standard	Case-series or superseded reference standards	Analysis with no sensitivity analysis
5	Expert opinion without explicit critical appraisal, or based on physiology, bench research or "first principles"	Expert opinion without explicit critical appraisal, or based on physiology, bench research or "first principles"	Expert opinion without explicit critical appraisal, or based on physiology, bench research or "first principles"	Expert opinion without explicit critical appraisal, or based on physiology, bench research or "first principles"	Expert opinion without explicit critical appraisal, or based on economic theory or "first principles"

Produced by Bob Phillips, Chris Ball, Dave Sackett, Doug Badenoch, Sharon Straus, Brian Haynes, Martin Dawes since November 1998. Updated by Jeremy Howick March 2009. RCT: Randomized clinical trials; SR: Systematic Review.

phase involved routine canine retraction and PGE1 was applied only on one side, which resulted in 1.6-fold faster movement on the treated side. The researchers did not find any adverse macroscopic effects either in the gum tissue or the alveolar bone. Only mild pain related to the dental movement was observed.

A second preliminary study was performed in 5 patients by Spielmann *et al*[10], with the common objective of assessing the effect of PGE1 on tooth movement. This differed from the previous study in that force was applied to the upper right and left premolars which were to be extracted later during the course of routine orthodontic treatment, and a reciprocal force was used. The method consisted of the local administration of anesthesia 0.1 mL of 0.01% (w/v) PGE1 solution in saline which was injected under the palatal mucoperiosteum to the test tooth and 0.1 mL saline palatal to the contralateral control tooth. Injections were repeated at weekly intervals.

On average the experimental teeth moved 3 times faster than the control teeth without any pathological changes.

Patil *et al*[11] in 2005, performed a clinical assay on 14 patients who were injected for three days with a dose of 1 g of PGE1 (3 g in total), using lidocaine as a vehicle substance in the distal buccal area of canines retracted with Niti open coils. The left side only received a vehicle substance as a control. The patients were monitored for 60 d and the authors concluded that following a minimal dose of PGE1 an increase in the rate of movement was

Table 2 Oxford centre for evidence-based medicine 2011 levels of evidence

Question	Step 1 (Level 1[1])	Step 2 (Level 2[1])	Step 3 (Level 3[1])	Step 4 (Level 4[1])	Step 5 (Level 5)
How common is the problem?	Local and current random sample surveys (or censuses)	Systematic review of surveys that allow matching to local circumstances[2]	Local non-random sample[2]	Case-series[2]	N/A
Is this diagnostic or monitoring test accurate? (Diagnosis)	Systematic review of cross sectional studies with consistently applied reference standard and blinding	Individual cross sectional studies with consistently applied reference standard and blinding	Non-consecutive studies, or studies without consistently applied reference standards[2]	Case-control studies, or "poor or non-independent reference standard[2]	Mechanism-based reasoning
What will happen if we do not add a therapy? (Prognosis)	Systematic review of inception cohort studies	Inception cohort studies	Cohort study or control arm of randomized trial[1]	Case-series or case-control studies, or poor quality prognostic cohort study[2]	N/A
What are the COMMON harms? (Treatment Harms)	Systematic review of randomized trials or n-of-1 trials	Randomized trial or observational study with dramatic effect	Non-randomized controlled cohort/follow-up study[2]	Case-series, case-control studies, or historically controlled studies[2]	Mechanism-based reasoning
What are the RARE harms? (Treatment Harms)	Systematic review of randomized trials, systematic review of nested case-control studies, nof-1 trial with the patient you are raising the question about, or observational study with dramatic effect	Individual randomized trial or (exceptionally) observational study with dramatic effect	Non-randomized controlled cohort/follow-up study (post-marketing surveillance) provided there are sufficient numbers to rule out a common harm. (For long-term harms the duration of follow-up must be sufficient)[2]	Case-series, case-control, or historically controlled studies[2]	Mechanism-based reasoning
Is this (early detection) test worthwhile?	Systematic review of randomized trials or n-of-1 trial	Randomized trial or (exceptionally) observational study with dramatic effect			
Is this (early detection) test worthwhile? (Screening)	Systematic review of randomized trials	Randomized trial	Non-randomized controlled cohort/follow-up study[2]	Case-series, case-control, or historically controlled studies[2]	Mechanism-based reasoning

[1]Level may be graded down on the basis of study quality, imprecision, indirectness (study PICO does not match questions PICO), because of inconsistency between studies, or because the absolute effect size is very small. Level may be graded up if there is a large or very large effect size; [2]As always, a systematic review is generally better than an individual study. OCEBM Levels of Evidence Working Group. "The Oxford 2011 Levels of Evidence". Oxford Centre for Evidence-Based Medicine. http://www.cebm.net/index.aspx?o=5653.

evident compared to the control group.

Vitamin D

The highest level of evidence 3B corresponds to an original Spanish article by Blanco *et al*[12]. The main objective of this study was to determine if a systemic dose of calcitrol supplement (0.25 μg), accelerates canine retraction movement over 60 d as compared to a control group.

Twenty patients (20 ± 5 years) whose canines were retracted using a stainless steel loop by applying a 75 g force when necessary every 15 d were included in this study. The subjects were randomly assigned to two groups: 10 received an oral dose of calcitrol 0.25 μg daily for 60 d and were monitored 10 times; the remaining 10 subjects acted as controls. An increased rate of movement was found in the experimental group ($P = 0.00028$). The researchers concluded that the average speed of movement was faster in the experimental group than in the control group.

PHYSICAL STIMULI

Vibratory forces

These are placed on the 4th level of the evidence scale, and the publications include a case series published by Kau in 2009[13] and 2010[14]. The main objective in the first publication was to report data resulting from use of the Acceledent System. In 2010, the clinical effects of the cyclical force generated by the device (Acceledent) on teeth and the average treatment time were reported. In addition, the levels of patient compliance and satisfaction were assessed. The sample size was the same for the two studies, 14 patients, 11 during leveling and alignment and 3 with space closure. The results of both of these studies were within the range of 0.526 mm of movement per week using Acceledent type I for 20 min daily during 6 consecutive months. Good patient compliance and satisfaction were observed.

Pulsed electromagnetic field

This is classified 3b evidence based on the study by Showkatbakhsh *et al*[15] in 2010, who showed that a pulsed electromagnetic field was capable of accelerating orthodontic tooth movement. The canines on one side in 10 patients who required canine retraction were exposed to a pulsed electromagnetic field (PEMF); the canines on the contralateral side in the same patients were not exposed to the PEMF. A circuit and a watch battery were used to

Table 3 Levels of evidence for therapies in orthodontics

Level	Therapy
	Prospective Meta analysis
	Meta analysis
1a	SR (with homogeneity) of RCTs
1b	Individual RCT
2a	SR (with homogeneity) of cohort studies
2b	Individual cohort study, CCTs and low quality RCT
3a	SR (with homogeneity) of case-control studies
3b	Individual Case-Control study, low quality cohort study and short time split mouth design
4	Case-series, low quality case-control study and non systematic review
5	Expert opinion

RCTs: Randomized clinical trials; SR: Systematic review; CCTs: Controlled clinical trials.

generate the PEMF (1 Hz). The generator was embedded in a removable device. Foil was used to prevent PEMF exposure in the control group. Showkatbakhsh *et al*[15] reported that the accumulative distance moved was significantly larger in the experimental group (5.0 ± 1.3 mm *vs* 3.5 6 ± 1.6 mm, $P \geqslant 0.001$) after 5 ± 0.6 mo.

Electrical current

Kim *et al*[16] demonstrated that an electrical current was capable of accelerating orthodontic tooth movement. Moreover, as only females (7) were included in this study, we do not know the effects of the electrical current in males. The electric appliance was set in the maxilla to provide a direct electric current of 20 microns. The maxillary canine on one side represented the experimental side, and the maxillary canine on the other side represented the control. The experimental canine received orthodontic force and an electric current. The control side received orthodontic force only. An electric current was applied to the experimental canines for 5 h daily, the authors showed that the accumulative distance moved was significantly larger in the experimental group after 1 mo (2.42 ± 0.26 mm *vs* 1.89 ± 0.27 mm). The electrical current was delivered to the mucosa of canines through a fixed electrical appliance assembly (20 MA, 5 h per day). This report is classified as 3b evidence based level.

Low level laser irradiation

Evidence based level 3b: Cruz *et al*[17] were the first to publish research on the effects of low level laser irradiation (LLLI) on the average speed of dental movement. The sample consisted of 11 patients who received a 150 g maxillary canine retraction force bilaterally for 2 mo, one side was irradiated and the other side was used as a control. Irradiation standards were wavelength 780 nm, power 20 mW, energy flow 2 J, energy density 5 J/cm^2, and total dose 8 J. The authors registered a 34% increase in the speed of dental movement on the experimental side compared to the control side[17].

Limpanichkul *et al*[18] used a different set of standards during laser application: 860 nm, 100 mW, 25 J/cm^2, 18.4 J around the experimental tooth (buccal mucosa, distal and palatal) 4 times over a month for a total dose of 294.4 J. The results did not show significant statistical differences between the experimental and control sides, concluding that the dose used (5 J/cm^2) was too low to achieve an increase in the rate of dental movement. To assess the effects of the laser (Ga-Al-As) during the retraction phase in canines, Youssef *et al*[19] irradiated the cervical, middle and the apical surface of the tooth on its buccal and palatal sides with 809 nm and 100 mW for 40 s; the total dose to the right upper and lower canines was 8 J (2 × 40 s at 100 mW), the left side was used as a control. The laser was applied using intervals of 0, 3, 7 and 14 d. The retraction coil was activated on day 21 for both sides. The study results showed a significant increase in movement rate for the irradiated canines when compared to the control[20].

Sousa *et al*[20] evaluated the effect of LLLI on the speed of orthodontic dental movement in 26 canines with retraction NiTi coil springs (150 g). 13 were irradiated (780 nm, 20 mW, 10 s, 5 J/ cm^2), and the other 13 were used as controls. The groups were followed for 4 mo with a total of 9 laser irradiations during that time. The authors concluded that the laser group, using the parameters described, showed an increased rate of orthodontic dental movement, and this could lead to a reduction in treatment time[21].

Evidence level 2b: Dominguez *et al*[21] in 2010 in a prospective cohort study, started at 5 mm crowding non-extraction and finished with a sample of 45 patients between 20 and 30 years old. The experimental group was irradiated at each appointment 1 mm away from the mucosa on the buccal and palatal sides, following the long axis of the tooth for 22 s on each surface. The control group did not receive laser irradiation.

The measurement unit used was days of treatment, the dosage and parameters of irradiation were: 830 nm, 100 mW, energy density 80 J/cm^2, an active laser point of 0.028 cm^2 and the energy was 2.2 J. These parameters allowed a reduction of 30% in the LLLI treated group during the total treatment time.

REGIONAL ACCELERATION PHENOMENA RELATED TECHNIQUES

Regional acceleration phenomena (RAP) healing is a complex physiologic process with dominant features involving accelerated bone turnover and decreases in regional bone densities. Following surgical wounding of cortical bone, RAP potentiates tissue reorganization and healing by a transient burst of localized hard and soft tissue remodeling[22-24].

Corticotomy

The majority of reports on corticotomy belong to level 4 in the scale of evidence.

In 1959, Kole[25] using the crowns of the teeth as

handles, believed that he was able to move the blocks of bone independently of each other as they were only connected by the less-dense medullary bone. He reported that combining orthodontics with corticotomy led to active tooth movement in adult orthodontic cases in 6 to 12 wk. The technique was known as "bony block". The interproximal corticotomy cuts were extended through the entire thickness of the cortical layer, just barely penetrating into the medullary bone. These vertical cuts were connected beyond the apices of the teeth with a horizontal osteotomy cut extending through the entire thickness of the alveolus, essentially creating blocks of bone in which one or more teeth were embedded.

Gantes *et al*[26] showed in 5 patients, that the corticotomy procedure caused minimal changes in the periodontal attachment apparatus. The surgical procedure included intracrevicular incisions and an elevation of buccal and lingual mucoperiosteal flaps. Buccal and lingual vertical grooves penetrating the cortical bone were then made between the roots. These grooves were extended from just below the interproximal alveolar bone margin to beyond the apex levels of the teeth. Buccal and lingual horizontal grooves joined the apical extensions of the vertical grooves. The orthodontic appliance was activated immediately upon wound closure.

In 1991, Suya *et al*[27] reported surgical orthodontic treatment of 395 adult Japanese patients with an improved surgical procedure that he referred to as "corticotomy-facilitated orthodontics."

The authors who have major quantities of scientific reports are the Wilcko[28-33] brothers starting in 2000 up to 2009 and these techniques are now known as Periodontally Accelerated Orthodontic and Osteogenic Techniques. Their reports show high success in acquiring accelerated dental movement which they attribute to an osteoclastic phase or catabolic phase from the regional acceleration phenomena. The Wilcko brothers introduced a technique combining alveolar corticotomies and bone grafting to prevent the risk of dehiscence and fenestration, while increasing the scope of orthodontic corrections. In this conventional approach, cortical incisions circumscribing the roots are made on both the buccal and palatal side following full thickness mucoperiosteal flaps. The bone graft is then placed facing the teeth to be moved and the flaps are then repositioned and sutured at the papilla.

This highly effective technique was also proven to be useful for the intrusion of overerupted molars as reported by Hwang *et al*[34] and Oliveira *et al*[35,36] and for incisive retraction by Germec *et al*[37].

In the study by Akay *et al*[38] all individuals received combined subapical corticotomy and a skeletal anchorage procedure, and intrusion forces of 200 to 300 g were applied to the attachments of each molar and both premolars for 12 to 15 wk. Their results indicated that the use of combined treatment with corticotomy and skeletal anchorage provided safe and noncompliant intrusion of posterior teeth in a short period and may be regarded as an alternative method for skeletal open bite correction in adults who reject orthognathic surgery.

Choo *et al*[39] performed a study to assess the results of surgical accelerated orthodontics in protrusive adults. 24 adults with maxillary or bimaxillary protrusion were treated with speedy surgical orthodontics, including maxillary perisegmental corticotomy followed by orthopedic en-mass retraction against C-palatal miniplate anchorage.

The authors found that the average total treatment time was 20 mo (range, 11-42 mo) and concluded that surgically accelerated orthodontics could be an excellent treatment alternative for adult patients with severe maxillary or bimaxillary protrusion.

In 2012, Bhat *et al*[40] knowing that significant acceleration in orthodontic tooth movement had been extensively reported studied a combination of selective alveolar decortication and bone grafting surgery. The latter was responsible for the increased scope of tooth movement and long-term improvement in the periodontium. A study was carried out in six patients diagnosed with class I malocclusion and bimaxillary protrusion. A modified corticotomy procedure was performed. Active orthodontic treatment began within 1 wk after surgery and the patients were followed up. The mean treatment time for these patients was 17.4 mo, and distalization of the canines was mostly completed within 8.5 mo.

Corticotomy studies level 3b: Fischer[41] evaluated the effectiveness of corticotomy comparing six consecutive patients presenting with bilaterally impacted canines. One canine was surgically exposed using a conventional surgical technique, while the contralateral canine was exposed using a corticotomy-assisted technique. The results showed a reduction in treatment time of 28%-33% for the corticotomy-assisted canines.

Aboul-Ela *et al*[42] evaluated 13 patients requiring the therapeutic extraction of the maxillary first premolars, with subsequent retraction of the maxillary canines. By using miniscrews as anchorage, canine retraction was initiated *via* closed nickel-titanium coil springs applying 150 g of force per side. Corticotomy-facilitated orthodontics was randomly assigned to one side of the maxillary arch of the canine-premolar region, and the other side served as the control. The average daily rate of canine retraction was significantly higher on the corticotomy side than the control side by 2-fold during the first 2 mo after corticotomy surgery. This rate of tooth movement declined to only 1.6-fold higher in the third month and to 1.06-fold by the end of the fourth month.

A study was conducted by Lee *et al*[43] on 65 Korean adult female patients with bimaxillary dentoalveolar protrusion to compare the orthodontic treatment outcomes of anterior segmental osteotomy and corticotomy-assisted orthodontic treatment. It was concluded that orthodontic treatment and corticotomy-assisted orthodontic treatment were indicated for patients with severe incisor proclination with normal basal bone position, although corticotomy-assisted orthodontic treatment had the advantage of shorter treatment duration. Anterior segmen-

tal osteotomy is recommended for bimaxillary dentoalveolar protrusion patients with gummy smile, basal bone prognathism, relatively normal incisor inclination, and relatively underdeveloped chin position.

Corticotomy study level 2b: Shoreibah *et al*[44] conducted a study to evaluate the effect of corticotomy-facilitated orthodontics (CFO) in adults using a further modified technique *vs* traditional therapy in orthodontic tooth movement. The sample included twenty orthodontic patients with moderate crowding of the lower anterior teeth which were randomly divided and treated with either a modified technique of corticotomy-facilitated orthodontic tooth movement (Group Ⅰ) or conventional orthodontic therapy (Group Ⅱ). The authors showed that there was a statistically significant difference between the two groups regarding treatment duration: 17.5 ± 2.8 wk in the CFO group and 49 ± 12.3 wk in the conventional orthodontic therapy group.

Piezocision

To overcome the disadvantages of other corticotomy techniques, Dibart *et al*[45] introduced a minimally invasive, flapless procedure combining piezo surgical cortical micro-incisions with selective tunneling that allows for bone or soft-tissue grafting. Due to their small size and precision, piezoelectric cutting inserts realize precise osteotomies without the risk of osteonecrosis[46]. The authors removed the lingual flap by performing only vestibular incisions, but the elevation of a flap prior to the corticotomy was maintained, thus only relatively reducing surgical time and postoperative discomfort.

Combined with proper treatment planning and a good understanding of the biological events involved, this novel technique can locally manipulate alveolar bone metabolism in order to obtain rapid and stable orthodontic results. Piezocision allows for rapid correction of severe malocclusions without the drawbacks of traumatic conventional corticotomy procedures. Previous reports and those published in 2011 are case series or single case reports (4th level of evidence) which conclude that piezocision is an effective therapy to reduce treatment time when compared to treatments such as Invisalign[47,48].

According to Uribe *et al*[49] corticotomies can potentially reduce the treatment time dramatically in patients who require a significant amount of molar protraction. The authors reported a single case (level 4), of a patient with agenesis of the lower second premolars, after the extraction of primary second molars, mucoperiostal flaps were elevated and interproximal vertical corticotomies were performed on the labial aspect of the mandibular molars with a piezo surgical microsaw. The vertical groove corticotomies were performed mesial to the first and second molars bilaterally and extended just below the crestal bone to the apex. Dried-freeze demineralized bone allograft was packed on the buccal surface covering the grooves and exposed labial cortical bone surface, including a dehiscence on the first molar. The edentulous zone was closed in ten months.

Dentoalveolar distraction

Dentoalveolar distraction (DAD) was performed by making monocortical perforations on alveolar bones around the canines, followed by distracting the canine using distractors.

The scientific literature shows the following case series and a single case report (evidence level 4).

According to Kişnişci *et al*[50] the concept of distraction osteogenesis for rapid orthodontic tooth movement is promising and feasible for clinical practice.

They reported a case series of eleven patients whose first premolars were extracted, and the buccal bone was carefully removed. After wound closure, a special orthopedic device was mounted and cemented to the first molar and canine teeth. Distraction started the same day at the rate of 0.4 mm twice a day and continued until adequate movement of the canine teeth was achieved.

According to Işeri *et al*[51] the dentoalveolar distraction technique is an innovative method that reduces overall orthodontic treatment time by nearly 50%. The authors conducted a study that consisted of 20 maxillary canines in 10 subjects, the first premolars were extracted, the dentoalveolar distraction surgical procedure was performed, and a custom-made intraoral, rigid, tooth-borne distraction device was put in place. The canines were moved rapidly into the extraction sites in 8 to 14 d, at a rate of 0.8 mm per day and full retraction of the canines was achieved in a mean time of 10.05 (± 2.01) d. The same results with the same sample characteristics were published by Akhare *et al*[52] in 2011.

Kurt *et al*[53] reported a 15-year-old skeletal and dental class Ⅱ female patient, with an overjet of 9 mm who was treated by DAD osteogenesis. A custom-made, rigid, tooth-borne intraoral distraction device was used for rapid canine retraction. Osteotomies surrounding the canines were performed to achieve rapid movement of the canines within the dentoalveolar segment, in compliance with distraction osteogenesis principles. The amount of canine retraction was 7.5 mm in 12 d at a rate of 0.625 mm per day.

Kisnisci *et al*[54] reported Dentoalveolar Transport Osteodistraction to distalized canines in 73 alveolar cleft cases. Overall management of selected cases with wider defects may also be optimized and simplified through the transport distraction of a tooth-bone segment. The osteotomy involves designing a partial-thickness bony segment of the transportation of a canine tooth to close the gap resulting from the extraction of the first premolar without a discontinuity defect.

Periodontal distraction

Periodontal distraction was performed by making vertical grooves on the mesial side of the first premolar extraction sockets followed by the same distraction techniq ue as used in DAD. Liou *et al*[55] performed the procedure in fifteen consecutive orthodontic patients, in which twenty-six canine distractions, including 15 upper and 11

lower canines, were carried out with custom made, tooth-borne, intraoral distraction devices. Right after the first premolar extraction, the interseptal bone distal to the canine was undermined with a bone bur, grooving vertically inside the extraction socket, along the buccal and lingual sides, and extending obliquely toward the base of the interseptal bone to weaken its resistance. The interseptal bone was not cut through mesiodistally toward the canine. The intraoral distraction device was delivered for canine distraction right after the first premolar extraction. It was activated 0.5 to 1 mm/d. The authors concluded that the periodontal ligament can be distracted just like the midpalatal suture in rapid palatal expansion. By using this concept, canines can be distracted distally 6.5 mm in 3 wk without significant complications.

Other case series (Level 4) reports are as follows: Gürgan *et al*[56] in 2005, during a 12 mo follow-up period, but without a control group, analyzed 36 maxillary canines until full retraction of the canines was achieved in 10.36 ± 1.93 d (range 8-14 d) at a rate of 0.8 mm/d using a custom-made intraoral rigid tooth-borne distraction device. The periodontal follow-up results allowed them to conclude that dentoalveolar distraction is an innovative technique with no unfavourable long-term effects on the gingival tissues of rapidly retracted canine teeth.

Sukurica *et al*[57] in a six month follow-up study, evaluated twenty canine retraction movements in eight patients. The distraction procedure was completed in 12 to 28 d (mean 14.65 ± 3.49 d). The distal displacement of the canines ranged from 3 to 8 mm (mean 5.35 ± 1.22 mm).

Kumar *et al*[58] concluded that canines can be rapidly retracted by periodontal ligament distraction without complications. The analysis was carried out in 16 upper canines in eight patients who required first premolar extractions. The upper first premolars were extracted and the interseptal bone distal to each canine was thinned and undermined surgically. Custom-built distractors were placed and activated immediately to distract the canines into the extraction spaces. The canines were retracted to proximal contact with the second premolars in 20.33 ± 1.87 d.

In a larger study of 43 canine teeth in 18 (seven male and 11 female) patients who required first premolar extractions conducted by Sayin *et al*[59], the canine retraction was carried out with teeth using semi-rigid, individual tooth-borne distractors. The maxillary canines were distalized an average of 5.76 mm with 11.47 degrees distal tipping. The mean distal movement of the mandibular canines was 3.5 mm with 7.16 degrees distal tipping.

In a split mouth randomized clinical trial without blinded outcome assessment (level 2b) involving 30 patients, Mowafy *et al*[60] evaluated the amount and time of canine retraction concomitant with periodontal ligament distraction using intermittent and continuous forces. For each patient, one side was randomly allocated to receive a screw-based dental distractor, and the other side received a continuous force coil spring distractor. The authors found that the average time needed for canine retraction was 5.3 ± 1.3 wk.

Mtdld technique

Case series: Evidence level 4: Vercellotti *et al*[61], developed a surgical-orthodontic technique [The monocortical tooth dislocation and ligament distraction (MTDLD) technique] to maximize the rapidity of dental movement and prevent damage to the periodontal tissues. During the procedure they performed a microsurgical corticotomy around each tooth, buccal monocortical tooth dislocation and palatal ligament distraction movement and the immediate application of biomechanical force. The report included 8 patients with malocclusion who underwent the procedure and the authors concluded that compared to traditional orthodontic therapy, the average treatment time with the MTDLD technique in the mandible and maxilla was reduced by 60% and 70%, respectively.

In 2011, Bertossi *et al*[62] performed piezosurgical bone cuts to 10 patients affected by different dental malformations to determine the effects of a shorter treatment time. This method (MTDLD technique) is simple, and performing osteotomic lines laterally and apically to the tooth radix on the bone has proved useful in reducing the treatment time. In addition, the technique is very easy to use and has a low incidence of complications. In 5 patients with dental ankylosis, dental repositioning was achieved within 18 to 25 d and in another 5 preoperative patients affected by maxillary hypoplasia and transverse maxillary diameter reduction, in 68 to 150 d.

In 2010, Kharkar *et al*[63] conducted a non-randomized pilot study. The aim of this study was to assess and evaluate the best approach to reduce the overall orthodontic treatment time by means of distraction osteogenesis. The sample consisted of six patients, comprising two groups, who were compared using two different surgical techniques: dento-alveolar distraction and periodontal distraction to bring about rapid canine retraction using a designed intra-oral distractor. Dento-alveolar distraction was superior to periodontal distraction in the time required for retraction, canine tipping, anchorage loss and amount of external root resorption. As a controlled clinical trial with a small sample this was classified as evidence level 3b.

Comparing acceleration techniques and amount of dental movement, Long *et al*[64] in 2013 conducted a systematic review with an evidence level 3 that included cases and control studies and concluded that corticotomy its an effective and safe method to accelerate dental movement in orthodontics. Alveolar or periodontal distractions are promising methods to promote orthodontic movement acceleration, but they lack enough convincing evidence to support them.

Alveocentesis (micro-osteoperforations)

Evidence level 4: Nicozisis[65] showed clinical examples of orthodontic treatments using propel, used in rotation, molar uprighting, Quicker Pre-surgical Orthodontics, intrusion and crowding. In addition, other reports which can be used successfully include, but are not limited to, TADs, Invisalign®, Sure Smile,® and conventional braces.

This includes the study by Teixeira *et al*[66] in 2010, and the results of both animal and clinical studies have demonstrated that the PROPEL System using the Alveocentesis technique decreases orthodontic treatment time by 50%-60% or more in combination with any type of orthodontic force.

Surgery first

The performance of surgery without orthodontic preparation (*i.e.*, "surgery first"), followed by regular postoperative dental alignment, was proposed by Nagasaka *et al*[67]. The authors used this approach to correct skeletal class Ⅲ malocclusion with the aid of skeletal anchorage system orthodontics. The total treatment time was noticeably reduced. In addition, preoperative profile worsening due to incisor decompensation was avoided and immediate profile improvement after surgery was greatly appreciated by the patient.

According to Liou *et al*[68], the advantages of the surgery-first approach are as follows: (1) the patient's chief complaint, dental function, and facial esthetics are achieved and improved at the beginning of treatment; (2) the entire treatment period is shortened to 1 to 1.5 years or less depending on the complexity of the orthodontic treatment; and (3) the phenomenon of postoperative accelerated orthodontic tooth movement reduces the difficulty and treatment time of orthodontic management in the surgery-first approach.

Liou *et al*[69] conducted a study in twenty-two adult patients, who received Le Fort I osteotomy of the maxilla and bilateral sagittal split of the mandible for dentofacial deformities. Crevicular fluid levels of serum alkaline phosphatase and C-terminal telopeptide of type I collagen were determined, as well as tooth mobility of the maxillary and mandibular incisors in these patients. The results support the hypothesis that the phenomenon of postoperative accelerated orthodontic tooth movement is due to the increase in osteoclastic activity and metabolic changes in the dentoalveolar caused by orthognathic surgery. The orthognathic surgery triggers 3 to 4 mo of higher osteoclastic activity and metabolic changes in the dentoalveolar postoperatively, which possibly accelerates postoperative orthodontic tooth movement.

Studies on surgery first are mainly case reports and case series (Evidence based level 4).

Uribe *et al*[49], described a 16-year-old female with a concave profile and class Ⅲ malocclusion, who received a surgical maxillary LeFort 1 advancement and completed her whole treatment within eight months. This was followed by a number of successful case reports that showed short treatment time for ortho- surgical cases using the surgery first approach in class two and three patients: Sugawara *et al*[70], Yu *et al*[71], Villegas *et al*[72] (asymmetrical class Ⅲ), Baek *et al*[73], and Oh *et al*[74].

Hernández-Alfaro *et al*[75] reported 2 cases successfully treated with bimaxillary surgery first. In patient 1, the total orthodontic treatment required 250 d. Arch settlement and leveling achieved a Class I relationship, with adequate root parallelism that was stable at follow-up 1 year later. For patient 2, the total orthodontic treatment lasted 185 d, after which an adequate Class I occlusion and an esthetically balanced profile was achieved.

In 2013, Hernández-Alfaro *et al*[76] reported treating forty-five patients with a surgery first approach. Selected cases presented with symmetrical skeletal malocclusions with no need for extractions or surgically assisted rapid palatal expansion. Standard orthognathic osteotomies were followed by buccal interdental corticotomies to amplify the regional acceleratory phenomenon. Miniscrews were placed for postoperative skeletal stabilization. Orthodontic treatment began 2 wk after surgery. Mean duration of orthodontic treatment was 37.8 wk (range, 24 to 52 wk). Orthodontic retention followed in all cases. An average of 22 orthodontic appointments (range, 14 to 29) occurred. The authors concluded that the surgery first approach significantly shortened total treatment time and was favorable in patients and orthodontists. Nevertheless, careful patient selection, precise treatment planning and fluent bidirectional feedback between the surgeon and the orthodontist are mandatory.

Evidence level 2b: Choi *et al*[77] in 2013, performed a prospective study to determine intervention outcomes in 24 standard and 32 surgery-first approaches for patients with skeletal class Ⅲ dentofacial deformity. In the surgery-first approach, a dental model was created and a novel preoperative orthodontic simulation of the standard presurgical orthodontic treatment was performed to determine the final occlusion between the maxilla and mandible. Changes in cephalometric landmarks were compared between the standard and surgery-first groups in the preoperative, immediate postoperative, and postoperative periods. The researchers found that a surgery-first approach without presurgical orthodontic treatment is possible and can give similar results to standard orthognathic surgery.

Corticision

"Corticision" was introduced as a supplemental dentoalveolar surgery in orthodontic therapy to achieve accelerated tooth movement with minimal surgical intervention. In this technique, a reinforced scalpel is used as a thin chisel to separate the interproximal cortices transmucosally without reflecting a flap[78].

In Young-Guk Park's[79] lecture (level 5), he described the procedure in detail: (1) in previously anesthetized subjects the surgical blade is inserted interproximally and parallel to the occlusal plane 5 mm apical from the tip of the papilla. The blade is tapped with a mallet to a depth of approximately 8 mm. The angle of the blade to changed to approximately 45 degrees apically and the blade is tapped to a depth of 10-12 mm. The blade is changed after four to five slices. The goal is to cut the cancellous bone between the roots to 50%-75% of the root length. To remove the blade, the blade and handle are grasped and the scalpel is worked up and down a few times before pulling the blade out. The blade is pulled

rather than the handle to avoid breaking the blade. Test the mobility of the teeth by forcibly trying to move them slightly. Apply orthodontic forces immediately. The patient is seen every two weeks and the teeth are forcibly mobilizing to induce minor trauma to extend the effect; and (2) according to Park, this is a minimally invasion technique to induce accelerated tooth movement by stimulating osteoblasts and bending alveolar bone that has been surgically separated.

According to Bondemark[80], there is no movement acceleration technique that provides strong evidence (at least two studies with high value of evidence: Randomized clinical study or a prospective study with a well-defined control group). Accordingly, it is necessary to have an alternative to classify the limited literature available on this particular subject, and randomized clinical trials on this topic must be developed.

Multiple reports on orthodontic acceleration in humans have been observed using an alternative evidence level scale and this a simple and accurate way of determining which technique is most effective. The highest level of evidence for a specific procedure to accelerate orthodontic dental movement up to October 2013, is for surgery first, followed by low level laser application and corticotomy located on level 2, recommendation grade b from this proposed scientific evidence scale. Nonetheless, there is a necessity for more studies with a higher level of evidence,considering that this teraphies are located on a moderate level of evidence.

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REVIEW

World health dilemmas: Orphan and rare diseases, orphan drugs and orphan patients

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Author contributions: Kontoghiorghe CN reviewed the ethical issues on orphan and other drug treatments; Andreou N reviewed the orphan drug topics; Constantinou K reviewed the disease profile in developed and developing countries; Kontoghiorghes GJ reviewed the issues related to iron chelating drugs, orphan drug development and the role of pharmaceutical companies and he also designed, wrote and edited the manuscript, which is part of a postgraduate project.

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Abstract

According to global annual estimates hunger/malnutrition is the major cause of death (36 of 62 million). Cardiovascular diseases and cancer (5.44 of 13.43 million) are the major causes of death in developed countries, while lower respiratory tract infections, human immunodeficiency virus infection/acquired immunodeficiency syndrome, diarrhoeal disease, malaria and tuberculosis (10.88 of 27.12 million) are the major causes of death in developing countries with more than 70% of deaths occurring in children. The majority of approximately 800 million people with other rare diseases, including 100000 children born with thalassaemia annually receive no treatment. There are major ethical dilemmas in dealing with global health issues such as poverty and the treatment of orphan and rare diseases. Of approximately 50000 drugs about 10% are orphan drugs, with annual sales of the latter approaching 100 billion USD. In comparison, the annual revenue in 2009 from the top 12 pharmaceutical companies in Western countries was 445 billion USD and the top drug, atorvastatin, reached 100 billion USD. In the same year, the total government expenditure for health in the developing countries was 410 billion USD with only 6%-7% having been received as aid from developed countries. Drugs cost the National Health Service in the United Kingdom more than 20 billion USD or 10% of the annual health budget. Uncontrollable drug prices and marketing policies affect global health budgets, clinical practice, patient safety and survival. Fines of 5.3 billion USD were imposed on two pharmaceutical companies in the United States, the regulatory authority in France was replaced and clinicians were charged with bribery in order to overcome recent illegal practises affecting patient care. High expenditure for drug development is mainly related to marketing costs. However, only 2 million USD was spent developing the drug deferiprone (L1) for thalassaemia up to the stage of multicentre clinical trials. The criteria for drug development, price levels and use needs to be readdressed to improve drug safety and minimise costs. New global health policies based on cheaper drugs can help the treatment of many categories of orphan and rare diseases and millions of orphan patients in developing and developed countries.

Key words: World health issues; Global diseases; Orphan drugs; Orphan diseases; Rare diseases; Orphan patients; Thalassaemia; Deferiprone; Deferasirox; Deferoxamine; Iron overload

Core tip: The major world health problems are related to poverty and other monetary health issues, including the supply of orphan drugs for the treatment of rare and orphan diseases. Differences in disease profile, disease burden and monetary health policies influence the mortality and morbidity rates of patients in developed and developing countries. The inexpensive developmental procedure of the iron chelating drug, deferiprone, used in thalassaemia is proposed as a paradigm for orphan and rare drug development. Improve-

ments in worldwide health policies including procedures for inexpensive drug development and alleviation of poverty could reduce the mortality and morbidity rates of patients worldwide.

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INTRODUCTION

The efforts of international organisations such as the World Health Organisation (WHO) and The United Nations Children's Fund, as well as many other national government organisations and non governmental organizations are continuously improving health standards worldwide and global health care is becoming a reality[1]. However, at the same time there are many challenges, ethical dilemmas and major issues related to health policies and strategies that still need to be addressed and resolved globally, including the treatment of patients in developing countries and patients worldwide with rare diseases. Major limiting factors for addressing such problems are the ability to provide successful treatments and the availability of financial resources[2,3].

Despite continuous medical progress in the treatment of diseases in the last few decades, the level of poverty and malnutrition, as well as the lack of health facilities and medicinal products are still considered the major factors leading to the high mortality and morbidity rates observed globally and mostly in developing countries[3]. In the developed countries, the disease profile classification affecting the mortality and morbidity rate is comparatively different with obesity, ageing and environmental pollution being considered as some of the major causes of many illnesses[4].

Monetary issues are very important in relation to the provision of health care and unless patients are self-sufficient financially, relevant decisions and priorities for selecting which disease, which drug and which patient to be treated are not yet fully clarified in each country or even in each hospital. Such dilemmas are more prevalent in developing countries, where financial resources are very limited. For example malnutrition and diarrhoea in infants and treatment of diseases such as malaria and human immunodeficiency virus infection/acquired immunodeficiency syndrome (HIV/AIDS) are priorities for long-term health strategies in the developing countries, whereas cancer, cardiac and neurological diseases are treatment priorities for most developed countries[1,2].

Among the major obstacles affecting the level of global health care is the availability and cost of medicinal drugs. There are many economic, ethical and other issues affecting the supply of drugs for different groups of patients in each country[5-7]. Within this context, regulatory procedure differences, preventative and diagnostic procedures, marketing influences and monetary factors can variably affect the treatment of patients in each country[8-10]. Many such treatments usually involve the use of vaccines, generic and sometimes new patented drugs.

In many cases, the progress of the disease and the outcome of the treatment may be affected because of wrong evaluations and choices regarding the available therapeutic options. Similarly, the risk/benefit assessment for the use of specific drugs is not in many cases clearly defined and in some cases is not necessary. For example, the widespread use of antibiotics for the treatment of the common flu is inappropriate because the common flu is caused by a viral infection. Other related issues are the toxicity of drugs, where the risks outweigh the benefits and can sometimes cause severe damage or can even be fatal.

Incentives for the development of new drugs, especially for diseases where effective treatments are not currently available, are considered a major challenge for academic and pharmaceutical industry researchers. In general, the development of new drugs is market driven and has little to do with rare diseases in the developed countries or serious tropical diseases in the developing countries. However, monetary incentives have been introduced in recent years in the United States, Japan and Europen Union (EU) countries, for the development of "orphan drugs" for "orphan diseases", where a small number of patients in these countries are affected by a rare condition in comparison to the general population. The motive in most cases for orphan drug development is the lucrative profit from monopolies of patented drugs and not humanitarian concerns for the well-being of the small group of patients with rare or orphan diseases in developed countries or the lack of treatment for patients in the developing countries (orphan patients)[11].

The expansion of the pharmaceutical industry in the developing countries and the local production of generic drugs are major advances in the treatment of local patients and the overall health levels in these countries[7]. However, despite the encouraging progress and the production of medicinal drugs in developing countries, many patients especially those with chronic conditions cannot afford the cost of even the locally developed generic drugs or newly available technologies[12].

A major obstacle in the introduction of new drugs for the treatment of any diseases is the anticipated high cost of drug development[13]. A further obstacle in the supply of new drugs in the developing countries is the high cost due to the drug monopolies implemented by world trade laws. It appears that such laws mostly benefit multinational pharmaceutical companies which are based in the developed countries[14].

Despite the fact that in many cases there are substantial improvements in the treatment of diseases due to the introduction of new patented drugs, there are also many other cases where the opposite result is observed. The latter may be caused by many factors including wrong decision making by those responsible for the approval and supply of the drugs due to misinformation regard-

ing drug safety and efficacy. Within this context there are many grey areas in the development and use of such new drugs, which in the long-term may worsen the treatment of affected patients and ultimately decrease the progress in the treatment of related diseases.

The design of new patented drugs, which is a major research task for pharmaceutical companies and academic institutions, usually involves many stages including the synthesis of many analogues, *in vitro* and *in vivo* screening studies and clinical trials. For example, in the case of the iron chelating drug, deferiprone (L1), which was invented and developed in academic institutions, more than one hundred analogues and other similar compounds have been designed and tested for possible application in the treatment of iron overload in thalassaemia and other diseases[15-18].

Despite the fact that thousands of new compounds are designed, tested and patented for possible application as new pharmaceuticals, only a very small number of these compounds reach the stage of investigational new drug (IND) or new medical entity and selected for further development.

Excessive costs for toxicological, carcinogenicity and other screening, clinical trials and post-marketing surveillance, as well as other costs such as patent fees make it impossible for individuals or academic institutions to proceed to the full development of a new drug. It is estimated that the cost for the introduction of a new drug from the stage of design to post-marketing monitoring is about 0.5 billion USD[19]. However, most of this expenditure is not related to the scientific evaluation and development of the drug, but due to its marketing. This procedure adds substantially to the price of a drug and to the overall public health spending, while at the same time it reduces the prospect of drug availability in developing countries[20]. In contrast, the development of academically based orphan drugs such as L1 can cost less than 5% of the above amount.

Present practices suggest that irrespective of how effective and safe new INDs might be, these are not likely to be developed, unless they are under patent monopoly and can potentially make huge profits following their registration and marketing. This issue is indicative of the current role of multinational pharmaceutical companies in societies, where profit is the major target for drug development and not the treatment of diseases. Pharmaceutical companies involved in the production of generic drugs are also trying to maximise profits. In the latter case, the profit levels are lower due to competition with other companies because of the absence of monopolies.

A paradigm which could affect future strategies in drug design, development and use are the iron chelating drugs. These drugs are primarily used for the iron removal treatment in thalassaemia and other transfusional iron loaded diseases such as myelodysplasia and sickle cell disease. In chemical terms, chelation can be considered a chemical reaction involving the formation of bonds between a metal ion and a chelator (Greek: χηλή-claw of a crab) resulting in a metal-chelator complex. A chelator can be a natural or synthetic chemical compound, or in this case a drug molecule capable of forming a heterocyclic ring with a metal ion as the closing member *i.e.*, like a crab holding the metal ion in its claw. In transfusional iron loading conditions the aim of the administration of iron chelating drugs is the binding and removal of excess toxic iron from the body[15-17].

HEALTH ISSUES AFFECTING GLOBAL MORBIDITY AND MORTALITY

Global health is related to a dynamic state of interactions, involving many factors and many players such as the WHO, local and governmental health authorities. Some of these factors include the availability of financial resources, the severity, transmission and extent of infectious, communicable and other diseases and the differences between ages and gender. In an ever expanding world population, major issues such as health provision and resource allocation have a major impact on human survival, morbidity, mortality and quality of life. Within this context, human activities such as monetary policies, wars, accidents and injuries, food production and distribution, health education, provision of medicines and health care as well as environmental pollution, infectious, chronic and genetic diseases, all interact and influence health outcomes in each country and worldwide[21].

Health models and schemes have been designed where diseases and patients have been included in different categories. At the same time, different strategies have been developed for addressing local and international health problems with one of the major limiting factors being the availability of financial resources. In all cases, financial resources for health care are limited and many classifications have been designed for outlining the importance, impact, morbidity, and mortality of each disease on a national and international scale (Table 1)[22-28].

In an attempt to prioritise the impact and severity of different diseases, including effects on the survival rate and quality of life of patients, several parameters have been introduced such as quality-adjusted life years (QALY), disability-adjusted life year (DALY) and years lived with disability (YLDs) which are mostly used in the developed countries for comparison among diseases and individual patient cases. QALY is a term referring to a calculated score for the comparison of different healthcare interventions which takes into account an average life expectancy and the quality of life or both. For example one year of perfect health is equal to 1 QALY, death is 0 QALY and a year of less than perfect health is scored between 0 and 1. A parameter related to health resource allocation is the cost/QALY, which is different for each intervention.

A DALY is another term used for measuring the amount of health lost due to a disease or injury. It is calculated as the present value of the future years of disability-free life that are lost as a result of premature death or disability occurring in a particular year. YLDs is another

Table 1 Main causes of death in developed and developing countries excluding malnutrition

People in developed countries (2002)	Millions	People in Developing countries (2002)	Millions	Globally (2011)	Millions
Heart attack	3.08	LRTI	2.81	Heart attack	7.00
Stroke	1.78	HIV/AIDS	2.55	Stroke	6.20
LTB cancer	0.61	Heart attacks	2.53	LRTI	3.20
LRTI	0.45	Infections at birth	1.78	COPD	3.00
COPD	0.42	Diarrhoeal disease	1.53	Diarrhoeal disease	1.90
Colon, rectal cancer	0.35	Stroke	1.45	HIV/AIDS	1.60
Diabetes	0.24	Malaria	1.25	Diabetes	1.50
Self inflicted injuries	0.23	Tuberculosis	0.96	LTB Cancer	1.40
Hypertensive heart disease	0.23	COPD	0.76	Road injuries	1.30
All causes	13.43	All causes	27.12		
Estimates 2006[1]		Malnutrition	36.00	All causes	62.00

[1]The total number of deaths globally in 2006 was estimated to be about 62 million, of which 32 million were related to hunger and malnutrition. COPD: Chronic obstructive pulmonary disease; LRTI: Lower respiratory tract infections; LTB cancer: Lung, tracheal and bronchial cancer. Adapted from ref[22-28].

term also used for health resource allocation of different interventions.

The main cause for the highest rate of mortality globally is hunger and malnutrition, which is found almost exclusively in the developing countries. It is estimated that one in twelve people worldwide is malnourished and that 58% of the total number of deaths is related to hunger or diseases due to deficiencies in micronutrients (Table 1). Despite the fact that world food production is adequate for feeding the entire human population, several causes such as insufficient food production, supply and distribution in the developing countries, as well as excess food use and waste in developed countries are the main reasons for the observed rate of malnutrition and human mortality today. In contrast, the high incidence of obesity, physical inactivity and smoking are some of the main causes for the high mortality and morbidity rates observed in relation to the most common diseases in the developed countries such as cardiovascular diseases, cancer and diabetes (Table 1)[22-28].

The cost/QALY for feeding the malnourished population in the developing countries is considered the lowest cost intervention globally. However, the adopted global food and health policies for solving this problem are insufficient and controversial. Similar controversial and ethical issues apply in the spectrum of diseases as well as related strategic policies aimed at increasing the survival and quality of life of people in developed and developing countries.

In relation to morbidity, the global outlook of diseases has general characteristics and individual variations between developed and developing countries. It is estimated that 13% of the global burden of disease is related to global mental health, surpassing both cardiovascular disease and cancer[29]. Depression, Alzheimer's disease and other dementias, epilepsy, schizophrenia, migraine, insomnia, multiple sclerosis, Parkinson's disease, alcohol dependence and other mental, neurological and substance-abuse disorders are included in this category of diseases[29]. Examples of the impact of diseases related to mental health is the annual rate of mortality from suicide which is estimated as 900000 people worldwide (200000 in China, 170000 in India, 140000 in high income countries) and the cost of dementia treatment, which for the United States alone has been estimated at 609 billion USD in 2009[29-31].

With the improvement of health practices and treatments there has been an overall increase in life span worldwide and a related increase in prognosis. Corresponding increases in expenditure have also been observed for many diseases in the developed countries, especially chronic diseases such as cardiovascular and neurological diseases, diabetes and cancer. The global burden of cancer for example, in 2002 was estimated at 10.9 million new cases, with 24.6 million persons alive with cancer (within 5 years of diagnosis) and 6.7 million deaths (61% in developing and 39% in developed countries). The incidence of cancer in men is as follows: lung followed by prostate, stomach, colorectal and liver cancer, and in women is breast followed by cervix uteri, colorectal, lung and stomach cancer. It has been suggested that the major causes of cancer in the United States are smoking (29%-31%), diet (20%-50%), infection (10%-20%), reproductive hormones (10%-20%), alcohol (4%-6%) and occupation (2%-4%)[32]. Cardiovascular disease was estimated to cause more than 17 million deaths worldwide in 2007 and this is projected to increase to 26 million in 2020, with 19 million in the developing and 7 million in the developed countries[25,26]. The major causes of cardiovascular disease are related to physical inactivity, tobacco use, high blood pressure, obesity, unhealthy diet, diabetes mellitus and alcohol use[33].

The estimated global burden of diabetes mellitus was 366 million people in 2012 with a projected increase to 552 million in 2030. Patients with prediabetes are estimated to reach 470 million by 2030 with a parallel increase in associated complications such as nephropathy, neuropathies and vascular complications[34].

In relation to transmitted diseases, a prominent position globally is HIV/AIDS with an estimated prevalence in 2007 of 33.2 million people living with HIV, including about 5% of adults in sub-Sarahan Africa and with an annual incidence of 2.5 million new cases and mortality of 2.1 million. Mortality due to HIV/AIDS has been reported to have decreased in 2011 due to prophylactic measures and new, more effective treatments (Table 1)[35,36].

Infectious diseases are one of the top groups of diseases with the highest morbidity and mortality rate affecting mainly patients in developing countries (Table 1). Neonatal and infant children are more susceptible to

Table 2 The largest health care companies in the world based on annual revenues

Rank	Company	Country	Total annual revenue (USD billions)
1	Johnson and Johnson	United States	61.90
2	Pfizer	United States	50.01
3	Roche	Switzerland	47.35
4	GlaxoSmithKline	United Kingdom	45.83
5	Novartis	Switzerland	44.27
6	Sanofi	France	41.99
7	Astra Zeneca	United Kingdom/Sweden	32.81
8	Abbott Laboratories	United States	30.76
9	Merck and Co.	United States	27.43
10	Bayer HealthCare	Germany	22.30
11	Eli Lilly	United States	21.84
12	Bristol-Myers Squibb	United States	18.81

The companies were ranked by revenue as of March 2010 according to their released 2009 annual reports.

such diseases, especially in poor areas with poor sanitary conditions and lack of clean water. More than 70% of deaths in this category are related to neonatal causes, pneumonia, diarrhea and malaria[28].

Many factors influence the morbidity and mortality rate for each disease with variations in different areas of the world. The major risk factors include malnutrition, sanitation, unsafe sex, tobacco, alcohol and illicit drugs, physical inactivity, obesity, hypertension and environmental pollution[37-39]. The global burden of diseases is in a dynamic state of continuous change, which can be monitored and hopefully will allow predictions and future strategies to be developed including the introduction of preventative measures and prognosis[22-39]. Such strategies can only be implemented if the necessary financial resources become available. Public spending on health, including the cost of drugs and services is under continuous evaluation and any adjustments may help to decrease current morbidity and mortality rates in many countries and also globally.

THE CONCEPTS OF ORPHAN DRUGS AND ORPHAN DISEASES

The attempts for global health coverage continue progressively and new national and international strategies for achieving this goal are steadily increasing with major successes[1-3,40,41]. The development of new drugs is part of this strategy and involves pharmaceutical companies mainly in Western countries where investment is available and revenues from sales could be colossal (Table 2).

A major challenge for global health coverage is also the development of treatment strategies for orphan and rare diseases and the development of orphan drugs. The term "orphan drugs" was introduced by governments of developed countries to help in the production and marketing of medicinal drugs by the pharmaceutical industry for patients suffering from rare conditions living in their own countries. This concept was based on monetary incentives and regulatory relaxations for attracting pharmaceutical companies to orphan drug production since it was assumed that the cost of developing and bringing to the market such a medicinal product cannot be recovered by the expected sales. On ethical grounds this concept is intended to help patients suffering from rare conditions to be entitled to the same quality of treatment as other patients with diseases affecting large numbers of the population.

Orphan drug legislation varies among the developed countries and was introduced at different times, first in the United States in 1983, Singapore in 1991, Japan in 1993, Australia in 1997 and in the EU in 2000. In the EU, an orphan medicinal product is intended for the diagnosis, prevention and treatment of an orphan disease with a prevalence of less than 5 affected per 10000 persons. The term orphan drug can also apply to a seriously debilitating condition even if its prevalence is more than 5 per 10000 persons. In the United States an orphan drug is intended for any rare disease with an incidence of less than 200000 persons. It is estimated that there are about 7000 orphan diseases ranging from genetic diseases such as thalassaemia to rare infections in the West such as malaria, tuberculosis and blinding trachoma. In addition, subsets of commoner diseases such as Crohn's disease of the oesophagus are also classified as orphan.

It is estimated that about 350 orphan drugs for 200 orphan diseases have been developed since 1983. Before the United States act of 1983 fewer than 40 products were developed, whereas between 1983 and 2009, the food and drug administration (FDA) approved 275 orphan drugs for 337 orphan indications and during the 2000s it was estimated that orphan products comprised 22% of all new molecular pharmaceutical entities[42]. Among the incentives for pharmaceutical companies in the United States, are market exclusivity for 7 years, grants of up to 30 million USD per annum, waiving of user fees (approximately 1.2 million USD for every application) paid to the FDA for review of the sponsor's application, tax incentives and easier to gain marketing approval. Similar conditions and relaxations are included in the EU legislation, but market exclusivity is for 10 years. It is estimated that global orphan drug sales have increased about 10% per year between 2005 and 2011, and are now approaching 100 billion USD annually[13].

Research in orphan diseases was until recently carried out mainly by academic institutions, biotech companies and smaller, specialty drug companies. Large pharmaceutical corporations have also lately taken interest, mainly for exploiting the orphan drug legislations by targeting sub-groups of common diseases[42].

Examples of a list of orphan-designated drug products with at least one marketing approval in the United States for a rare disease indication are shown in Table 3[43-45]. The drugs approved are mostly related to various cancers and other conditions with low prevalence in the developed countries. Marketing approval in the United States was also provided for orphan-designated drug products for both common and rare disease indications

Table 3 Examples of orphan-designated drug products with at least one marketing approval in the United States for a rare disease indication

Drug product name	Orphan indications
Alglucerase injection	Replacement therapy in Gaucher's disease
Alitretinoin	Acute promyelocytic leukemia
Alpha1-Proteinase Inhibitor	Cystic fibrosis
Ambrisentan	Idiopathic pulmonary fibrosis
4-Aminosalicylic acid	Crohn's disease
Amifostine	Chemoprotective agent in cancer
Anagrelide	Polycythemia vera
Anti-tac (human)	Prevention of acute graft-*vs*-host disease
Arsenic trioxide	Multiple myeloma, MDS, CML, CLL
Atovaquone	*Toxoplasma gondii* encephalitis
Azacitidine	Acute myeloid leukemia
Beractant	Newborn infants with pneumonia
Bosentan	Idiopathic pulmonary fibrosis
Busulfan	Primary brain malignancies
Calfactant	Acute respiratory distress syndrome
Canakinumab	Juvenile idiopathic arthritis
Capsaicin	Erythromelalgia
Cladribine	Non-Hodgkin's lymphoma, CLL, AML
Clofarabine	Acute myelogenous leukemia
Coagulation factor Ⅶa	Bleeding in Glanzmann thrombasthenia
Cysteamine hydrochloride	Huntington's disease
Cytarabine	Gliomas
Daunorubicin liposomal	Acute myeloid leukemia
Decitabine	Sickle cell anemia, CML, AML
Eculizumab	Dermatomyositis
Epoprostenol	Replacement of heparin in hemodialysis patients
Filgrastim	Myelodysplastic syndrome and AIDS
Fludarabine phosphate	Non-Hodgkins lymphoma
Heme arginate	Myelodysplastic syndromes
Idarubicin	AML in pediatrics, MDS and CML
Ifosfamide	Bone and soft tissue sarcomas
Iloprost solution for infusion	Heparin-associated thrombocytopenia
Indium^{111} pentetreotide	Neuroendocrine tumors
Interferon gamma-1b	Idiopathic pulmonary fibrosis
Lenalidomide	Mantle cell lymphoma and CLL
Levocarnitine	Pediatric cardiomyopathy
Mecasermin	Amyotrophic lateral sclerosis
Mecasermin rinfabate	Burns that require hospitalization
Melphalan	Cutaneous melanoma
Mesna	Inhibition of the urotoxic effects
Miglustat	Neurological manifestations
Mitomycin-C	Refractory glaucoma
Mycophenolate mofetil	Pemphigus vulgaris
Nilotinib	Gastrointestinal stromal tumors
Nitazoxanide	Intestinal amebiasis
Nitisinone	Alkaptonuria
Nitric oxide	Acute respiratory distress syndrome
Pentostatin	Cutaneous T-cell lymphoma and CLL
Porfimer sodium	Cholangiocarcinoma
Pralatrexate	Diffuse large B-cell lymphoma
Primaquine phosphate	*Pneumocystis carinii* pneumonia
Protein C concentrate	Replacement therapy in protein C deficiency
Procarbazine hydrochloride	Malignant glioma
Quinine sulfate	Non *Plasmodium falciparum* malaria
Rapamycin (mTOR) inhibitor	Tuberous sclerosis complex
Rifabutin	*Mycobacterium avium* disease
Riluzole	Huntington's disease
Sermorelin acetate	Induction of ovulation in women
Sodium phenylbutyrate	Sickling disorders
Sodium thiosulfate	Platinum-induced ototoxicity
Somatropin	Induction of ovulation in women with infertility
Succimer	Mercury toxicity and kidney stones
Synthetic human secretin	Diagnostic procedures in pancreatic carcinoma
Synthetic porcine secretin	Diagnostic procedures in pancreatic carcinoma
Temozolomide	Advanced metastatic melanoma.
Tetrabenazine	Moderate/severe tardive dyskinesia
Thalidomide	Graft *vs* host disease in BMT
Topotecan HCl liposomal	Gliomas
Tretinoin	Acute and chronic leukemia
Trimetrexate	Metastatic carcinomas
Vorinostat	Multiple myeloma and mesothelioma

ALL: Acute lymphoblastic leukaemia; AML: Acute myeloid leukaemia; CML: Chronic myeloid leukemia; CLL: Chronic lymphocytic leukaemia; MDS: Myelodysplastic syndrome; BMT: Bone marrow transplantation. Adapted from references[43-45].

as shown in the examples in Table 4[43-45]. In this group of orphan-designated drug products, new formulations and new indications of commonly used drugs (*e.g.*, doxorubicin, bleomycin and cyclosporine) have been included (Table 4)[43-45]. A list of drugs approved for rare diseases in the EU is also shown in Table 5[46]. The list includes many drugs intended in most cases for the treatment of various cancers, infectious diseases and other conditions with low prevalence in the EU (Table 5)[46]. It is estimated that there are about 2000 orphan diseases in the EU and 6500 in the United States[46,47].

The orphan diseases with the highest number of drug designations and most orphan drug approvals are shown in Table 6[48,49]. With the exception of malaria, which is a very rare condition in developed countries, but a major problem in developing countries, all other 13 diseases are rare diseases found in developed countries (Table 6)[48,49]. It is estimated that 11 tropical diseases related to helminth, protozoan and bacterial infections affect about 800 million people in developing countries, excluding malaria, communicable, environmental and genetic diseases (Table 7). Overall, the neglected tropical and other diseases found almost exclusively in developing countries are extremely rare in developed countries and are not a priority for orphan drug development in developed countries (Table 7). It is evident that the orphan drug and orphan disease concepts are mostly based on monetary considerations and not an effort for the treatment or elimination of rare and neglected diseases with high incidence in the developing countries. Patients suffering with neglected tropical and other rare diseases in developing countries can be considered as orphan patients. Similarly, as budgetary limitations on health are expanding in the developed countries the concept of orphan patients is also adopted for patients with rare and other diseases in the developed countries.

DILEMMAS IN MEDICAL ETHICS AND ORPHAN PATIENTS

Present Western philosophies and medical ethics are based upon monetary concerns, which affect local and global health levels. For example, enough food is pro-

Table 4 Examples of orphan-designated drug products with marketing approvals in the United States for both common and rare disease indication

Drug product name	Orphan indications
Adalimumab	Paediatric Crohn's disease
Aldesleukin	Primary immunodeficiency disease
Allopurinol	*Ex vivo* preservation of kidneys for transplants
Aminosidine	Tuberculosis and *Mycobacterium avium*
Azathioprine	Graft-*vs*-host disease
Aztreonam	Improvement of symptoms in bronchiectasis
Bevacizumab	Ovarian, stomach and pancreatic cancer
Bleomycin sulphate	Pancreatic cancer
Cetuximab	Pancreatic cancer
Cisplatin liposomal	Osteogenic sarcoma metastatic to the lung
Colchicine	Behcet's Syndrome
L-Cycloserine	Gaucher's disease
Cyclosporine	Prophylaxis and treatment of GVH disease
Cyclosporine A implant	Prevention of rejection in cornea transplant
Cyclosporine liposomal	Bronchiolitis obliterans
Doxorubicin	Hepatocellular carcinoma
Doxorubicin HCl liposomal	Soft tissue sarcomas
Doxorubicin nanoparticles	Hepatocellular carcinoma
Eflornithine HCl	*Pneumocystis carinii* pneumonia in AIDS
Epoetin alpha	Myelodysplastic syndrome
Erlotinib HCl	Malignant gliomas
Etidronate disodium	Degenerative metabolic bone disease
Everolimus	Gastroenteropancreatic tumors
Histrelin	Acute intermittent and other porphyries
Immuneglobulin	Juvenile rheumatoid arthritis
Infliximab	Chronic sarcoidosis
Interferon alfa-2a	Esophageal carcinoma
Peginterferon alfa-2a	Chronic myelogenous leukemia
Interferon alfa-2b	Ovarian carcinoma, brain tumors
Peginterferon alfa-2b	Chronic delta hepatitis
Metronidazole (topical)	Perioral dermatitis
Metronidazole	Pouchitis
N-acetylcysteine	Acute liver failure
Paclitaxel	Pancreatic cancer
Paclitaxel aqueous gel	Esophageal and brain cancer
Paclitaxel micellar	Ovarian cancer
Paclitaxel protein-bound	Stage ⅡB to Ⅳ melanoma
Sorafenib	Stage ⅡB through stage Ⅳ melanoma
Ribavirin	Haemorrhagic fever with renal syndrome
Rituximab	Immune thrombocytopenic purpura
Thiotepa	Haematopoietic stem cell transplantation
Tranexamic acid	Hereditary angioneurotic edema
Urofollitropin	Initiation and re-initiation of spermatogenesis
Ursodiol	Cystic fibrosis liver disease

GVH: Graft versus host. Adapted from ref[43-45].

duced to feed the whole world population, but large quantities are wasted or destroyed based on existing market policies. This allows millions of people to die from hunger and malnutrition. Similarly, market policies are also partly responsible for the limited success in the effort to prevent or eliminate many diseases and for the lack of basic medicines in developing countries. In general, access to drugs and treatments and health levels for each individual and each country depends on their ability to pay. This happens even in developed countries for example in deciding by clinical boards who can receive a kidney, heart, liver and other transplants or hip replacement or cardiac surgery. Similar dilemmas exist in deciding who can be treated with haemodialysis machines or by new, but very expensive drugs for the treatment of cancer and other serious conditions. Within this context the vast majority of orphan patients are in the developing countries, but there are also many orphan patients in developed countries, where treatments may be available, but patients have no access to them due to limited availability and health resources[22-27].

There are many conflicting interests, ethical and other issues affecting the healthcare of each individual at local and global levels. Healthcare strategies and policies are developed based upon different approaches, influences and philosophies. The ultimate decisions affecting healthcare resource allocation rely on government policies and legislations, which are influenced by political groups, commercial interests, patient groups, and other society groups in general[2].

Government policy in most countries relies on political dogmas between the capitalist approach suggesting that healthcare is another way of spending money and if people cannot afford it that is their bad luck, whereas in the socialist approach it is suggested that the distribution of healthcare is a matter of social justice and all individuals should be treated as equals. Many developed countries are using the utilitarian approach on healthcare resource allocation, which is for the greatest good for the greatest number of people and is measured by QALY. However, there are many dilemmas in resource allocation on healthcare related to QALY measurements, where terms such as good and quality of life have not been fully defined. For example there are age related issues, where treating younger patients will save more years of life, or social worth issues where contribution to the society may be considered as a morally relevant factor, or personal responsibility where individuals such as smokers and obese people are personally responsible for their ill health.

Health economics are increasingly becoming a major part of healthcare and medical education with a major emphasis on better allocation of resources by minimising costs and maximising healthcare output in the primary state control and run sector. This sector is the main healthcare provider in most countries and allocates the funds from taxes. Cost benefit, effectiveness and utility analysis are a major part of healthcare strategies since it is becoming increasingly clear that there are not enough professionals and not enough money to provide a comprehensive state controlled healthcare service in many countries. For example it was estimated in 2010 that medicines alone cost the National Health Service in the United Kingdom more than £13 billion per annum, which accounts for around 10% of the overall health budget[50].

In many developed countries expert independent committees have been instituted to tackle healthcare problems and design healthcare strategies. An example is the United Kingdom national institute for health and clinical excellence (NICE), which uses QALYs to determine which treatments are most suitable for each disease. Accordingly, clinicians use the advice of NICE to decide which treatments to prescribe their patients[50,51]. However, despite the fact that such efforts are necessary the

Table 5 List of drugs approved for rare diseases in Europe

Rare disease category	Drug product name (Indication)
Leukaemias, lymphomas and related diseases	Histamine dihydrochloride and Decitabine (AML) Ofatumumab (CLL) Nilotinib (CML) Mercaptopurine and Clofarabine (ALL) Cladribine (Hairy cell leukaemia) Ponatinib (Philadelphia chromosome positive ALL and CML) Dasatinib (CML and AML) Azacitidine (Myelodysplastic syndromes, CML, AML) Bosutinib (Philadelphia chromosome positive CML) Nelarabine (T-cell ALL and lymphoma) Brentuximab vedotin (Hodgkin lymphoma and anaplastic large cell lymphoma) Ruxolitinib (Primary and other myelofibrosis cases) Plerixafor (Lymphoma and multiple myeloma)
Carcinomas and related diseases	Sorafenib tosylate (Hepatocellular carcinoma, renal cell carcinoma) Mitotane (Adrenal cortical carcinoma) Mifamurtide (Osteosarcoma) Temsirolimus (Renal cell carcinoma and mantle cell lymphoma) Trabectedin (Soft tissue sarcoma, liposarcoma, ovarian cancer) 5-Aminole-vulinic-acid hydrochloride (Malignant glioma) Thalidomide and Lenalidomide (Multiple myeloma)
Chelating drugs and haemoglobinopathy related diseases	Deferoxamine, Deferiprone and Deferasirox (Iron overload in beta thalassaemia) Dexrazoxane (Anthracycline extravasation) Hydroxycarbamide (Sickle Cell Syndrome) Zinc acetate dehydrate (Wilson's disease) Eculizumab (Proxysmal nocturnal haemoglobinuria and haemolytic uraemia)
Pulmonary hypertension	Bosentan monohydrate (Pulmonary arterial and systemic sclerosis) Iloprost (Primary pulmonary hypertension) Ambrisentan and Sildanafil citrate (Pulmonary arterial hypertension)
Cystic fibrosis	Mannitol, Aztreonam and Ivacaftor (Cystic fibrosis) Tobramycin (Pseudomonas aeruginosa in Cystic Fibrosis)
Enzymes used as drugs	Velaglucerase alpha (Type 1 Gaucher disease) Alpha-glucosidase (Pompe disease) Galsulfase (Mucopolysaccharidosis VI) Idursulfase (Hunter syndrome) Proteolytic enzymes enriched in bromelain (Burns)
Drugs used in other rare conditions	Carglumic acid (Hyperammonaemia due to n-acetylglutamate synthase deficiency) Betaine anhydrous (Homocystinuria) Stiripentol (Severe myoclonic epilepsy in infancy) Pirfenidone (Idiopathic Pulmonary Fibrosis) Icatibant acetate (Hereditary angioedema) Amifampridine (Lambert-Eaton myasthenic syndrome) Alipogene tiparvovec (Familial lipoprotein lipase deficiency) Mecasermin (Growth failure in primary insulin-like growth factor 1 deficiency) Rufinamide (Lennox Gastaut syndrome) Sapropterin dihydrochloride (Phenylketonuria and tetrahydropterin deficiency) Romiplostim (Immune thrombocytopenic purpura) Nitisinone (Hereditary tyrosinemia type 1) Ibuprofen (Patent ductus arteriosus) Caffeine citrate (Primary apnea) Hydrocortisone (Adrenal insufficiency) Zicotide (Chronic pain) Teduglutide (Short Bowel Syndrome) Pasireotide (Cushing's disease) Thiotepa (Haematopoietic progenitor cell transplantation) Everolimus (Tuberous sclerosis complex) Tafamidis (Transthyretin amyloidosis) Anargelide hydrochloride (Essential thrombocythaemia) Miglustat (Type 1 Gaucher disease and Niemann-Pick type C disease)

ALL: Acute lymphoblastic leukaemia; AML: Acute myeloid leukaemia; CML: Chronic myeloid leukemia; CLL: Chronic lymphocytic leukaemia. Adapted from ref[46].

task is colossal considering that there are so many medicinal products available including 50000 drugs. These efforts are also limited by many conflicting interests such as conflicting literature, rivalry between pharmaceutical companies, rivalry between academics, differences between regulatory authorities, different priorities by health authorities, different patient group interests and society concerns[5,7,9,52]. The decisions by NICE are based on several factors including the guidance from ministers on the resources available, the clinical needs of patients in relation to other available technologies, the National Health System's priorities, the broad balance between benefits and costs and the potential impact on other National Health System's resources.

The ultimate decision to choose which treatments can be prescribed for the patients is made by the clinician or group of clinicians in charge. Despite the fact that treatment decisions by clinicians are expected to be guided by the Hippocratic oath, which broadly suggests that the treatment regimen to be followed should be according to the doctor's ability and judgment for the benefit of patients, many other factors and influences are involved. For example, the United Kingdom General Medical Council states that doctors should provide effective treatments based on the best available evidence, but also making efficient use of the resources available. On the other hand, thousands of pharmaceutical company representatives are continuously lobbying clinicians and other related groups to influence their decision in choosing appropriate treatments in favour of their products (Figure 1)[5,20].

One of the most important factors influencing healthcare economics and resource allocation is the cost of drugs. Patient requirements for drugs, the role and effects of pharmaceutical companies on drug pricing, efficacy and safety are in most cases being evaluated by expert committees. Within this context, pharmacoeconomics is a new expanding scientific area, influencing decision making of major healthcare resource allocation organisations such as NICE, the National Institute for Health in the United States and similar national organisations in many other countries worldwide.

DRUG DEVELOPMENT AND LARGE PHARMACEUTICAL COMPANIES

The lucrative market of pharmaceuticals and the patent monopolies of new drugs is a major contributory factor

Table 6 Orphan diseases with the most orphan drug approvals

Disease	Drug designation	Drugs approved
AIDS	57	8
Acute myeloid leukaemia	34	5
Ovarian cancer	34	4
Multiple myeloma	32	6
Glioma	29	4
Chronic myelogenous leukaemia	19	4
Acute lymphoblastic leukaemia	17	6
Pneumocystis carinii pneumonia	15	5
Respiratory distress syndrome, infant	14	6
Multiple sclerosis	14	5
Growth hormone deficiency	13	9
Idiopathic pulmonary hypertension	12	4
Kaposi's sarcoma	11	5
Malaria	11	4

Adapted from ref[48,49]. AIDS: Acquired immunodeficiency syndrome.

Table 7 Examples of neglected tropical and other diseases in developing countries

Disease categories	Diseases
Genetic diseases	Thalassaemias, sickle cell disease
Helminth infections	Ascariasis, hookworm, trichuriasis, schistosomiasis, lymphatic, filariasis, onchocerciasis, dracunculiasis
Protozoan infections	Human african trypanosomiasis chagas disease, leishmaniasis
Bacterial infections	Buruli ulcer, leprosy, trachoma
Environmental poisoning	Arsenate toxicity, bantou siderosis, mining industry, nuclear industry
Communicable and other diseases	HIV/AIDS, tuberculosis, malaria

HIV/AIDS: Human immunodeficiency virus infection/acquired immunodeficiency syndrome.

to the national economy and income of the most affluent developed countries. The revenue of the twelve top multinational pharmaceutical companies exceeded 445 billion USD in 2009 (Table 2). It should be noted that in the same year the total government expenditure for health in the developing countries was 410 billion USD[2]. Only, 6-7% of this expenditure was received as foreign aid from developed countries. For example, the global health fund of the United States over 6 years (2009-2014) was 63 billion USD[21]. The biggest selling drug of all time is the statin, Lipitor (atorvastatin), from the United States pharmaceutical company Pfizer with lifetime sales of 100 billion USD, until patent expiration in 2011[53].

Six of the twelve top pharmaceutical companies including the top two are based in the United States, two in Switzerland, two in the United Kingdom (one jointly with Sweden) and one each in France and Germany (Table 2). Rarely such companies are involved in the development of orphan drugs, unless they are familiar with the market potential and the income is similar to the non-orphan drugs. An example of an orphan drug is deferasirox (DFRA), which is marketed by one of the top twelve companies (Novartis) and used in the treatment of iron overload in thalassaemia and intended for many other iron loaded conditions[54].

Despite the fact that the standard regulatory authority procedures and laboratory tests needed for drug approval may differ slightly between organisations such as the United States FDA and EU European medicines agency (EMA), the major aspects of screening are based on similar preclinical and clinical testing. In general these procedures involve preclinical testing and usually four distinct clinical phases carried out over many years.

Drug design and development is usually undertaken by pharmaceutical companies in developed countries, due to suspected high expenditure requirements. The drug discovery period can take on average about 10 years and in general involves the design and screening of a large number of known chemical compounds of different classes from chemical libraries. Computer aided technology is a new method of drug design and development. Such methods involve, among others, the mimicking of existing drugs and introduction of structural modifications which may lead to higher efficacy and lower toxicity. However, such approaches are limited due to the complexity of the biological and physiological systems, which cannot be theoretically fully evaluated. Following the identification of leading groups of compounds, new chemical compounds are synthesised and screened to select the most promising ones for further evaluation and development. The screening process for the identification of a new product is tedious and success is limited. For example in the pharmaceutical company Hoechst during the period between 1972 to 1985, out of the 120000 new compounds synthesised and tested, it has been possible to launch only 15 new products.

Structure/activity correlation and preclinical safety testing can take 2-6 years and clinical safety and efficacy studies can take 6-10 years. In the case of orphan drugs, the preclinical and clinical testing period is shorter and involves fewer procedures. The testing requirements and regulatory approval for orphan drugs appear to be different between the United States, EU and other countries. For example, the iron chelating drug L1 was first approved in India in 1994, in the EU and other countries in 1999 and the United States in 2011[55].

The preclinical testing of new drugs involves *in vitro* and *in vivo* experiments. Chemical, biochemical and cell studies, including mutagenicity studies, are carried out during the *in vitro* testing. In the *in vivo* testing, animal studies in at least three different mammalian species using different doses are assessed to evaluate preliminary information on efficacy, absorption, distribution, metabolism and excretion (ADME), pharmacokinetics and toxicity. Following this initial screening procedure the drug could be selected for further evaluation as an investigational new drug (IND).

The clinical testing can be initiated, provided the preclinical testing is satisfactory. In the clinical testing, the initial studies (Phase I) involve in general the administration of low sub-therapeutic doses of the IND to a small number of (*e.g.*, 10-15) normal volunteers to establish whether the drug is tolerated and to derive

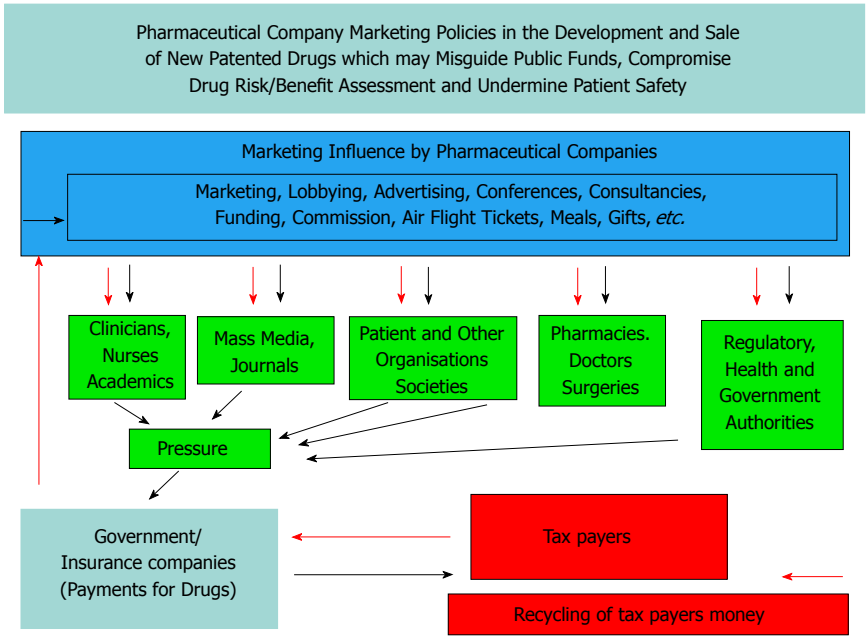

Figure 1 Ethical issues arising from the influence of pharmaceutical companies. A diagram of a theoretical model describing the marketing influence of pharmaceutical companies on various sectors and organisations in relation to new patented drugs and its effect on public spending.

pharmacokinetic, pharmacodynamic and metabolic data. Provided that the preliminary data are satisfactory, further clinical studies involving a larger number of normal volunteers (*e.g.*, 20-100) or sometimes patients are carried out using escalating doses in order to establish a therapeutic dose range, and to assess safety and tolerability. More information on pharmacokinetic and pharmacodynamic data are gathered at the higher dose levels and other parameters are investigated such as the effect of food on drug absorption if the drug is planned to be administered orally.

Phase Ⅱ clinical trials involve a larger number (*e.g.*, 300) of normal volunteers and patients, with the major aim of establishing safety and efficacy ranges. The identification of a therapeutic dose range and further pharmacokinetic studies are also carried out at this phase.

Phase Ⅲ studies involve many more patients (*e.g.*, 1000) over longer periods, usually in randomised controlled multicentre clinical trials, with the main aim of comparing the new drug to the current gold standard drug. The duration of the studies depends on the medical condition and is much longer for chronic conditions. In addition to long-term toxicity monitoring in this phase, other toxicity parameters such as carcinogenicity and drug interaction studies are also carried out. Provided the phase Ⅲ trials are successful and the safety and efficacy results are satisfactory then all the data from chemistry to human studies are submitted and the drug could be registered and approved by the regulatory authorities for marketing. Specific recommendations on the labeling for directions of use and list of adverse effects are included in the marketed product. Following approval by the regulatory authorities, the assessment of the drug may be extended to different patient subgroups and diseases.

Post-marketing surveillance, which is also known as phase Ⅳ trials or pharmacovigilance, can be introduced to detect any rare or long-term adverse effects in a much larger patient population, which was not available during the previous clinical trial phases. Many drugs have been withdrawn or their use restricted due to toxicity at this stage, *e.g.*, rofecoxib[56]. In many cases phase Ⅴ, which is also sometimes referred to as translational research, is now being used to compare the overall effect of the new treatment with other treatments and its impact on public health and the general patient population[20].

It should be noted that there are many variations in the clinical testing procedures and phases for each drug that can affect the length of studies. These include for example the seriousness of a condition, the categories of patients that can be treated, the concomitant use of other drugs and new requirements on safety and efficacy that may be requested by the regulatory authorities. Similarly, other parameters that can also contribute to the length of development and marketing of a drug are whether the drug is needed for urgent treatments or untreated conditions or orphan diseases. In all cases of the introduction of a new drug, a risk/benefit assessment and comparison with the standard treatment should prevail. However, in most cases the major factor for the development and sale of a new drug is financial gain through patent monopoly and intensive marketing.

A major issue in drug development and subsequent use is the level of toxicity. Despite the fact that all drugs have toxic side effects and each individual's susceptibility to toxic side effects is different, no major effort or procedures have been instituted in the drug development or subsequent post-marketing period for studying and reducing or reversing the cause of drug toxicities. The same lack of interest also applies to the design of diagnostic and prophylactic procedures for reducing the incidence of drug toxicities for generic drugs.

Emphasis in both the case of new and generic drugs involve marketing methods for increasing sales, but not improvements for patient safety such as protocols for

minimizing or preventing the toxic side effects or the production of drug antidotes. Within this context, the introduction of patents of new drug formulations, which in most cases have similar efficacy and toxicity to the old formulations, is another area exploited by multinational pharmaceutical companies for making additional huge profits due to the patent monopoly restrictions.

It is estimated that there are about 50000 different drugs available globally, which cause about 8000 different toxic side effects most of which affect patients to a different degree. It is also estimated that approximately 5% of the patients in hospitals are receiving treatments related to the toxic side effects of drugs. There are also many patients affected by toxicity due to impure drugs, idiosyncratic reactions, drug interactions and organ function complications.

The investigation of individual variations in drug response such as pharmacogenomics and metabolomics, as well as the introduction of drug combinations are fast expanding areas in drug development and for the application of personalised medicine.

THALASSAEMIA AND ORPHAN IRON CHELATING DRUGS

Thalassaemia and sickle cell disease are some of over 200 inherited haemoglobinopathies which are included in the category of orphan diseases. Similar to other orphan diseases there is a need for the development of orphan drugs for their treatment. Within this context, iron chelating drugs which are essential for the treatment and long-term survival of thalassaemia patients are classified as orphan drugs. The development of iron chelating drugs requires a basic understanding of iron metabolic processes and methods targeting the effective elimination of iron[17].

Iron is an essential element required by many biological processes and for normal physiological function. There are many iron metabolic disorders affecting millions of people. Iron deficiency is thought to affect a quarter of the world's population, but is not considered to be a severe condition and can in most cases be treated with iron supplements. In contrast, iron overload is considered to be the most common metal toxicity condition worldwide, with severe implications in morbidity and mortality[57]. The most common conditions of iron overload are caused by increased gastrointestinal iron absorption (primary haemochromatosis) or multiple red blood cell transfusions (secondary haemochromatosis) or a combination of these two processes. While in normal individuals there is a balance of iron intake and iron loss, in iron overloaded patients there is a net intake of iron. The rate of net iron intake in patients with primary haemochromatosis is slower (about 2-6 mg/d) than that of transfused patients with secondary haemochromatosis (about 15-30 mg/d)[57].

It is estimated that in general patients with refractory anaemias such as β-thalassaemia are regularly transfused with 1-3 units (1 unit = 200 mg of iron) of red blood cells every 1-4 wk. An excess of 100-125 g of iron, which is equivalent to about 500 units of red blood cells can be stored in the body of β-thalassaemia patients by the time they reach adulthood. Most of the iron accumulated from transfusions and increased iron absorption is not excreted, but is stored as excess, mostly intracellularly in the form of the iron storage proteins ferritin and especially haemosiderin. The organs mostly affected are the liver, heart, spleen and endocrine system. The damage to these and other organs due to iron overload toxicity is detectable when about 50-100 units of red blood cells have been transfused and is so extensive that in many cases it can become irreversible and fatal, unless iron chelation therapy is commenced[58].

Transfusional iron overload in refractory anaemias has the highest mortality and morbidity rate worldwide by comparison to any other form of metal overloading condition. The most seriously affected group of transfused patients are those with β-thalassaemia, but there are also increasing numbers in other transfused categories of patients affected such as sickle cell anaemia and myelodysplasia. In the latter two conditions, iron chelation therapy may not be critical since the overall iron accumulation in most cases is less and accordingly the rate of mortality caused by iron overload toxicity is lower in comparison to β-thalassaemia.

The epidemiological data of regularly transfused patients with different conditions has not yet been fully evaluated. It is estimated, for example, that 100000 children are born with β-thalassaemia and about the same number with sickle cell disease each year[59]. The latter is prominent in the black populations in African countries and their descendants in other continents, especially North America. A smaller number of patients with sickle cell disease can also be found in other countries such as those of the Middle East. β-thalassaemia is found mainly in countries in the Mediterranean area, Middle East and South East Asia. More than 80% of β-thalassaemia patients live in South East Asia and the Middle East and less than 10% worldwide receive adequate transfusions and iron chelation therapy mainly due to the unaffordable cost of treatment. The vast majority of β-thalassaemia patients in developing countries are left to die untreated.

In countries like Cyprus, β-thalassaemia heterozygotes are estimated to be 16% of the population, whereas in India it is 1%-10% of the population depending on the area. The incidence of thalassaemia in Western Europe and North American countries is very low and is related to the flow of immigrants from endemic areas. In Western Europe and North America, β-thalassaemia is considered an "orphan disease" because of the small number of patients in comparison to the rest of the population, who are not carriers of the β-thalassaemia gene[59]. There is a 25% chance that a couple who are heterozygotes, carriers of the β-thalassaemia gene, can give birth to a β-thalassaemia major child. β-Thalassaemia major patients can only survive if they receive regular red blood cell transfusions from normal haemoglobin blood

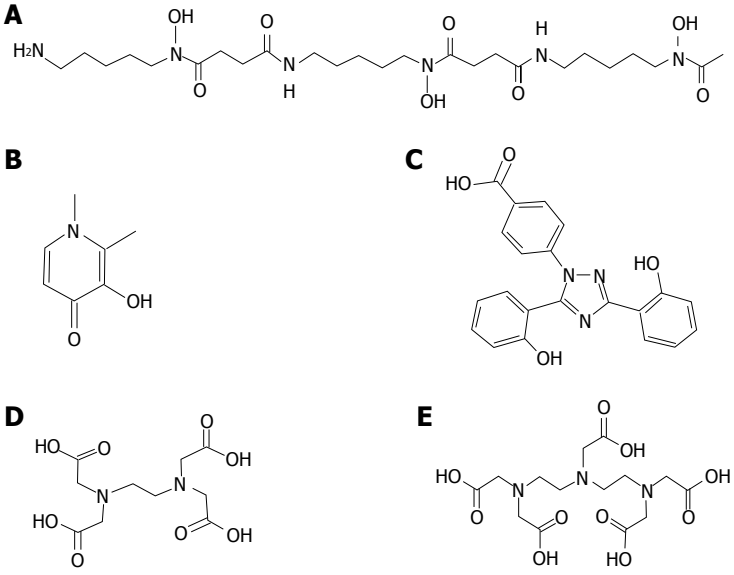

Figure 2 The chemical structure of chelators in clinical use. The chemical structures of the three orphan iron chelating drugs (A-C) which are used for the treatment of iron overload in thalassaemia and two other chelators used in other conditions (D and E): (A) deferoxamine (DF); (B) deferiprone (L1), (C) deferasirox (DFRA), (D) ethylenediaminetetraacetic acid (EDTA) and (E) diethylenetriaminepentaacetic acid (DTPA).

donors in order to replace their ineffective erythrocytes, which contain an abnormal non-functional haemoglobin, unable to transport oxygen to the tissues.

Heart failure as a result of iron overload toxicity from repeated red blood cell transfusions has been until recently the major cause of death in β-thalassaemia patients, which usually occurs before the age of twenty years[58]. This can be minimised or prevented with iron chelation therapy, especially since L1 was introduced[60]. In many developed countries, bone marrow transplantation is used instead of transfusions and iron chelation therapy. This method of treatment is usually applied to a small percentage of mostly very young β-thalassaemia patients and incurs a mortality rate of about 5%-9%[61]. Most global efforts are focused on the prevention of births of β-thalassaemia children using prenatal diagnosis and antenatal procedures[59].

Iron chelating drugs are primarily used for the treatment of iron overload in thalassaemia, which is considered an "orphan disease" in the EU, United States and many other developed and developing countries. The general objective for the design and development of iron chelating drugs for the worldwide treatment of iron overload in thalassaemia and other diseases is that they should be inexpensive, orally effective and non-toxic. However, in addition to thalassaemia and other diseases of transfusional iron overload there has recently been an increased interest in the use of chelating drugs as the main, alternative or adjuvant therapy in many non-iron loaded diseases. The design of iron chelating drugs for clinical applications other than the treatment of iron overload requires different selection criteria and developmental procedures[62].

There are three main iron chelating drugs in clinical use at present, which are used for the treatment of transfusional iron overload, namely deferoxamine (DF), L1 and DFRA (Figure 2). The chelating drugs ethylenediaminetetraacecetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA) have been previously used in transfusional iron overload, but were not selective for iron and are currently used for the detoxification of other toxic metals[63]. In particular, EDTA is used in millions of patients in alternative medicine worldwide and DTPA in the detoxification of plutonium and other radionucleotides in the nuclear industry[55,64,65].

There are many controversial, ethical and other issues surrounding the design strategies that led to the discovery and development of the oral chelating drugs L1 and DFRA, their comparison to the injectable drug DF and their current therapeutic use in developed and developing countries.

CONTROVERSIES IN THE USE OF IRON CHELATING DRUGS

The historic development of the iron chelating drugs including efficacy, toxicity, cost and ethical aspects as well as other issues can be followed chronologically through the published medical literature. Within this context, there have been many exchanges questioning the role of pharmaceutical companies and academia in the development of iron chelation therapy.

Both DF and L1 are generic drugs, whereas DFRA is a relatively new patented drug and all three are marketed in many countries worldwide. Deferoxamine has been the mainstay of iron chelation therapy since the 1960's and was marketed by Ciba Geigy (now Novartis). Both DF and L1 are currently marketed by several companies worldwide. In contrast, DFRA is marketed by Novartis worldwide except India, where a local company (Cipla) has registered DFRA based on a local court ruling and is sold in India at a price 60-times cheaper than in the EU and United States (Scrip 2008: S00990226)[66,67].

Deferiprone was invented in 1981 in the United Kingdom and selected as a leading chelating compound out of about 150 related analogues and other compounds[15-18]. It was developed as an academic initiative, which at the first stages was mostly financed by the thalassaemia patient's

organisation in the United Kingdom. Following many preclinical studies, L1 received approval for clinical trials from the local hospital ethical committee and the Department of Health of the United Kingdom in 1986[68,69]. The encouraging clinical trial results in the United Kingdom prompted the expansion of the clinical trials in many European countries, in Canada and in India[70-74]. The multicenter clinical trials were part of an academic initiative involving mainly thalassaemia patients who were unable to receive DF due to toxicity, low compliance or both. Deferiprone was first registered in India in 1994 and then in the EU, Asia and other countries in 1999 and the United States in 2011[55]. The cost of development of L1 in the United Kingdom up to the stage of multicentre clinical trials was less than 2 million USD.

There were many controversies and exchanges regarding L1, amongst academics and between academics and pharmaceutical companies, with one case reaching the mass media involving a Canadian pharmaceutical company (Apotex) and an academic clinician claiming liver toxicity during clinical trials[75-77]. Similarly, embryogenic and other toxicity caused by L1 in non-iron loaded animals was claimed by the company Ciba Geigy (now Novartis), which was then manufacturing DF[78]. In both cases the toxicity was not confirmed by other groups studying L1 in clinical trials with thalassaemia patients and in iron loaded animals[79-81]. These controversies and exchanges highlight the marketing tactics of competing pharmaceutical companies on drugs, which in this case may appear to have been planned to delay the use of L1 until a new owned drug, in this case DFRA was introduced. Similar exchanges were published regarding the possible use of L1 in thalassaemia patients in developing countries, since the high price of DF was prohibitive for the vast majority of patients, who could not afford chelation therapy[81,82].

Deferasirox is a known compound developed by Novartis, which was selected out of a library of more than 1000 compounds and was provisionally approved for clinical use in 2005. The preclinical studies with DFRA were limited and did not address many of the *in vitro* and *in vivo* efficacy and toxicity studies, which were carried out and published during the preclinical development of L1. Initial clinical trials with DFRA in 2003 showed that negative iron balance was not achieved at the then maximum dose of 30 mg/kg per day[83]. Higher doses were not then recommended because of the prospect of renal damage[83]. The maximum dose has now been increased to 40 mg/kg per day for better efficacy, but with increasing prospects of toxicity[84]. Regarding the cost of chelating drugs, it is estimated that in the EU and other developed countries the approximate yearly cost of effective dose protocols for a 50 kg man at 45 mg/kg, 5 d per week on DF is about 5000 euros (excluding needles and pumps, *etc.*) and for L1 at 80 mg/kg per day, 8000 euros. For DFRA at 30 mg/kg per day the cost is about 33000 euros and at 40 mg/kg per day about 60000 euros. The cost of the L1/DFO combination, *e.g.*, the ICOC L1/DFO combination protocol of using L1 during the day (80-100 mg/kg per day) and of subcutaneous DF (40-50 mg/kg at least 3-4 d/wk) is 11000-14000 euros. Deferiprone is sold in India at a price around 4 times cheaper than comparison in Europe and is even sold at a much cheaper price by a local company in Thailand. The same applies for DFRA, where the sale price in India is more than 60 times lower than in Europe and the United States (Scrip 2008: S00990226).

It is estimated that DFRA is costing about 150 euros per patient per year to manufacture and is sold at 60000 euros, with most of the cost claimed as marketing expenses[20]. Both L1 and DFRA can be sold at a price more than 100 times cheaper by non-profit organizations in developing countries, but such initiatives are still lacking, despite the fact that 80% of the thalassaemia patients die untreated in these countries.

Regarding the treatment aspects of transfusional iron overload in thalassaemia, DF is considered to be effective when administered subcutaneously or intravenously at 40-60 mg/kg per day and for oral L1 at 75-100 mg/kg per day, whereas the efficacy of oral DFRA at 20-40 mg/kg per day is still questionable and under investigation[83,85-89]. Iron removal from the heart, which is the main cause of mortality in iron loaded thalassaemia patients, is achieved mainly by L1 and to a lesser extent by DF and DFRA. The latter two chelators appear to be more effective in iron removal from the liver.

In terms of efficacy, the International Committee on Chelation (ICOC) L1/ DF combination protocol is considered to be the most effective because it reduces iron load to normal range body iron store levels[90-93]. The introduction of the ICOC L1/DF combination was an academic initiative based on a model for improving efficacy and reducing toxicity. Many drug combinations are widely used for other diseases in clinical practice without the need for regulatory approval. In contrast, almost all pharmaceutical companies disapprove of such synergistic combinations unless the drugs prescribed are both owned by the same company. Monotherapy with DF, L1 or DFRA are generally less effective than the ICOC L1/ DF combination protocol and only L1 has been shown to reduce and maintain thalassaemia patients' body iron store levels to normal physiological ranges[92,94].

Another major issue in relation to the efficacy of chelation therapy is compliance, which is reduced in patients receiving DF due to the long term 8-24 h daily injections, whereas it is much higher in patients receiving the oral chelators L1 and especially DFRA. In addition to efficacy and compliance, the toxicity properties of the chelating drugs are also a major factor in the overall risk/benefit assessment. Optimal chelation therapy is based on the selection of the appropriate chelating drug(s) and chelation protocol in each condition and for each patient. As shown in many other conditions, each patient appears to have an individual ADME, sensitivity, toxicity and efficacy profile for each chelating drug. Such individual response profiles are currently investigated within the framework of different parameters such as pharmacogenomics, metabolomics and proteomics, which are essential requirements for designing personalised medicine

protocols[85,95].

Different toxic side effects have been reported during the 50 year long-term use of DF and 27 years for L1 in different cohorts of patients. However, in contrast to the other two chelators and despite the fact that toxicity in the medical literature is rarely reported, it is evident from post-marketing reports that DFRA has one of the highest rates of fatal toxicity among new patented drugs, which is associated with renal, hepatic, gastrointestinal and bone marrow failure[96-102]. In FDA and user community toxicity monitoring, 4113 fatalities in patients using DFRA were reported in 2012. Previous FDA individual case based reports of 2474 deaths, suggest that there has been uncontrollable and indiscriminate use of DFRA in many categories of iron loaded and non-iron loaded patients, as well as a lack of toxicity monitoring and absence of prophylactic measures[100]. In a post-marketing report in 2009, DFRA was listed as the second most frequent suspect drug in reported patient deaths following rosiglitazone, with 1320 and 1354 fatalities, respectively[98]. In an EMA meeting the same year, an 11.7% mortality rate (1935 cases out of 16514 patients) was reported and a warning was issued that DFRA's toxicity is likely to increase when the maximum recommended dose increases from 30 to 40 mg/kg per day[99].

A few fatalities have also been reported in the case of DF mainly in relation to mucormycosis, yersiniasis and bone marrow aplasia[103-105]. Similarly, a few cases of fatal agranulocytosis have also been reported with L1, especially in patients who did not adhere to mandatory weekly or fortnightly blood counts, which are used as prophylaxis[106-108].

In addition to these fatal cases, there were many other non-fatal, but serious toxic side effects reported for DFRA which were mainly associated with damage to kidneys, liver, bone marrow and gastrointestinal tract, as well as several other organs such as ocular and auditory abnormalities and skin rashes[109-113].

The toxic side effects in the case of DF were ocular and auditory abnormalities, administration site (injection) related complications of mainly topical or sometimes systemic toxicity and yersiniasis[17]. The toxic side effects of L1 included in addition to agranulocytosis, cases of neutropenia, joint and musculoskeletal pain, gastric intolerance and zinc deficiency, all of which may affect about 5%-10% of patients[17].

The selection of optimal iron chelation therapy protocols in thalassaemia mainly includes the use of DF and L1. The overall risk/benefit assessment suggests that for the vast majority of patients the combination of L1 with DF is the most effective, least toxic protocol and where it is appropriately used it has resulted in a substantial decrease in morbidity and mortality in different thalassaemia patient cohorts[114-116]. This therapeutic approach has changed thalassaemia from a fatal to a chronic disease[116]. Similarly, the ICOC L1/DF combination protocol has resulted in normalisation of the iron stores in thalassaemia patients, which can in most cases be maintained by low doses of L1 monotherapy[90-95]. Despite these encouraging findings, many patients are still treated with DFRA monotherapy which has a better compliance but lower efficacy and a much higher risk/benefit ratio in comparison to DF, L1 and their combination. Only a small proportion of patients can benefit from the use of DFRA, especially those having toxicity complications with DF and L1[85,89-95]. Many patients are also using DF and L1 monotherapy, where iron overload is stabilised, but not eliminated to the extent of achieving normal range body iron store levels.

The variation in iron chelation treatments in different hospitals, countries and overall worldwide, reflects the influence of doctors on drug selection, patient safety and treatment outcome. This variation also reflects the marketing influence of pharmaceutical companies on doctors, health authorities and governments, and highlights the factors influencing the ability of doctors and health authorities to identify and select appropriate therapy protocols for patients[20].

Further developments in the area of iron chelation therapy are the ongoing clinical trials and uncontrolled clinical use of DFRA in other categories of transfused iron loaded patients such as myelodysplasia and sickle cell disease, and in non-transfused iron loaded patients such as hereditary haemochromatosis, thalassaemia intermedia, and post-transplanted thalassaemia patients. This wider use approach and development undermines patient safety and increases the risk/benefit ratio since any long or short-term benefits on morbidity and mortality from the use of DFRA in other categories of patients and especially in myelodysplasia and sickle cell disease patients are questionable and not yet confirmed[117,118]. Similarly, venesection in hereditary haemochromatosis and post-transplanted thalassaemia patients is a much safer and inexpensive procedure in comparison to DFRA treatment. The same applies to many non-transfused thalassaemia intermedia patients, where chelation therapy with L1 and DF is also safer than DFRA[119]. The wider use of DFRA in the above conditions indicates the pharmaceutical company's marketing potential and the influence it exerts on the risk/benefit assessment of chelation therapy by individual clinicians.

The use of iron chelating drugs in patients with non-iron loaded diseases is another expanding area attracting a lot of interest among clinical investigators and pharmaceutical companies. Similarly, the application of iron chelating drugs could include rare and tropical diseases where no effective treatment is currently available[120-126]. The safety of L1 in non-iron loaded patient categories seems to be higher in comparison to DF and DFRA, as shown from many short and long-term clinical trials in neurodegenerative, renal, infectious and other diseases[62,127]. In contrast, fatal and other serious toxic side effects have been observed in clinical trials using DF, in non-iron loaded patients such as in mucormycosis and rheumatoid arthritis patients[104]. Similarly, in one of the FDA related reports it was estimated that of the 2474 individual fatal cases reported for DFRA at least 500 were not related to transfusional iron overload, but included

cancer, cardiovascular, neurological and other patients with normal iron store levels[100]. Within this context, L1 appears from the various clinical trials with non-iron loaded patients to be relatively safe and promising for wider clinical use as a main, alternative or adjuvant therapy in many diseases, and as a pharmaceutical antioxidant[62,128,129].

The evaluation methods and the controversies which were identified in relation to the development and use of chelating drugs may help in the introduction of new approaches and strategies for the design, development and use of orphan and other drugs. Such academic approaches may benefit the treatment of millions of patients with many other conditions worldwide. Within this context, the paradigm of the design and development of chelating drugs, and especially L1, which was based on academic initiatives, may help to illustrate the need for new strategies in drug development. The use of this approach can increase the accessibility to new drugs due to low drug prices and decrease the morbidity and mortality observed in many diseases in both developed and developing countries.

Drug development based on academic initiatives can minimise costs and increase the prospects of the introduction of new drugs, which can be applied in the treatment of many diseases in both the developing and developed countries. At present the high prices of new patented drugs which are the result of high costs in drug development are not affordable for the vast majority of patients.

In addition to new strategies for the reduction of costs in the production of orphan and other drugs, several other initiatives can be taken to improve patient treatments worldwide. These include further research challenges involving better understanding of the heterogeneity in the underlying mechanisms of disease processes, inter-patient variability in drug responses and better risk/benefit assessment procedures.

CONTROVERSIES IN DRUG DEVELOPMENT AND PATIENT SAFETY

In almost all cases of the development and marketing of new patented drugs, which are mostly undertaken by private multinational pharmaceutical companies, a number of marketing strategies are developed for maximising sales and profit for their product such as advertisements, publications, lobbying and conferences (Figure 1). Within this context, a large marketing plan is constructed involving among others the recruitment of consultants who are usually internationally known influential academics in clinical departments of public hospitals mainly in Western Europe and the United States. These consultants are usually involved in seeding clinical trials and the promotion of their new drug product in publications, conferences, patient organisations and regulatory authorities[20,130].

The market plan also involves arrangements where company representatives personally visit all clinicians who have jurisdiction over potential patient/customers, with the prospect of advertising and promoting their drug to be used by their patients and in most cases offering in exchange different forms of support or gifts ranging from a meal, to the covering of expenses for their participation in conferences where the drug is promoted, consultancies, grants and other benefits (Figure 1).

In many countries with no legal restrictions, pharmaceutical company representatives often offer a percentage of the sale of the drug they promote to clinicians and to other influential persons in health or regulatory authorities. A different marketing strategy is to offer a clinician a "compensation" for "enrolling" patients in clinical trials, with the prospect of the patients involved to remain on the treatment and the government health authorities to pay huge sums of money for the continuation of the expensive new treatments (Figure 1). For example, 5000 euros is paid to the clinicians in charge of the enrolment of each patient for post-marketing monitoring of one of the new patented drugs in Greece.

A number of cases involving bribery of clinicians by pharmaceutical companies have reached the courts, but corruption is so extensive and out of control that in one court case in Germany a limit of 10000 euros was allowed as a donation for such activities for private clinicians, most of whom are working for the National Health System[131-135]. It should be noted that all the benefits paid to the clinicians for drug promotion are included in the cost of drugs as marketing expenses and are paid by the tax payers through the government health authorities (Figure 1). In developed or other countries with legal restrictions on the influence of private organisations, financial support by pharmaceutical companies is provided indirectly through donations to academic, patient, charity and other organisations associated with the supporters or promoters of their drug (Figure 1).

Within the framework of new drug development and marketing, multinational pharmaceutical companies can provide clinicians with financial support for different events and in some cases for research projects and clinical trials involving their drug, under a secrecy agreement[136-139]. Usually, only positive results are allowed to be published by the investigators financed by the pharmaceutical company, which are under the scrutiny of their marketing, legal and medical writer's department[137-140]. Similarly, reports of toxic side effects and studies of low or no efficacy are rarely published. Within this context, pharmaceutical companies influence academic research and academic affairs, including the impact factor of journals, the citations of articles and the citations for authors. Academic consultants of pharmaceutical companies can also influence publications of competing new or generic drugs, by serving in editorial boards or as referees. The unbiased role of journals is also questioned, since almost all journals are businesses and dependent on income from the pharmaceutical industry including advertisements, reprints and conferences[140,141].

Similar influences can also be exerted in other aca-

demic platforms and in medical conferences, especially when the pharmaceutical company is a sponsor. The number of conferences organised and sponsored by a single pharmaceutical company for the promotion of their drug is continuously increasing and support for independent conferences where competing drugs are presented is continuously decreasing.

In addition to targeting clinicians, a similar marketing strategy by pharmaceutical company representatives is also directed to other individuals or organisations, which may influence the sale of their drugs such as patient organisations, academic societies, regulatory authorities and other governmental bodies (Figure 1). For example, most of the conferences related to medicine including the expenses for participation by clinicians, nurses and patients are supported by multinational pharmaceutical companies introducing new drugs. Similarly, pharmaceutical companies are the major sponsors for medical societies, patients' organisations and selected medical departments in academic institutions (Figure 1).

Despite the fact that in some developed countries a number of restrictions have been introduced to reduce the influence of pharmaceutical companies in academic and public institutions, the influence is still evident. Such influences may not appear to be on a direct personal level, but are exerted indirectly, for example by donations or grants to academic institutions and societies[137-141].

In general, the marketing approach is different for each pharmaceutical company, for each country and each "customer" and depends mainly on the local conditions and the potential market scale. This can be illustrated by the variability on the sale figures and the use of the three iron chelating drugs in different countries, hospitals and individual doctors.

The marketing influence of multinational pharmaceutical companies for safeguarding the sale of their new drugs and for generating new income worldwide is evident from many recent events such as the health scare in relation to the spread of A H1N1 influenza and other similar viruses, where vaccines worth billions of dollars were sold worldwide[142]. It should be noted that national governments and the WHO endorsed and advertised the campaign for the pharmaceutical companies to sell and distribute these vaccines worldwide, even when these were not licensed[66,142]. Following the scare warnings some of the national and international advisors in the decision making panels for the approval and supply of the vaccines were later identified also to be consultants for the pharmaceutical companies selling the vaccines[66,142]. This and similar examples illustrate the need for the creation of independent public expert committees, *e.g.*, like NICE in the United Kingdom for safeguarding the rights of patients and for protecting and distributing national health resources according to the health needs of patients and not according to the influences of pharmaceutical companies[143].

There are many other grey areas in the development and sale of pharmaceuticals involving marketing tactics and strategies which are jointly developed between pharmaceutical companies such as price fixing, market sharing, market exclusivity and other arrangements between the companies selling the generic drugs and the new drug or competing drugs. In all of these cases public funds and spending on drugs is misappropriated and pharmaceutical companies make huge profits, which reduce health resources and in many cases may also affect patient safety[20].

The protection and domination plan of the patent monopoly for a new drug product in specific diseases is another major part of the marketing strategy of the patent holder pharmaceutical companies. This may involve the discrediting of competing drugs for example through publication of possible adverse effects by academics supporting their drug or through legal conflicts in relation to exclusivity, or on therapeutic claims made by similar drugs. Other methods used include the acquirement of new patents and delaying tactics in the development of an investigational new drug that may influence the sale of their drug. These marketing and other tactics are pursued not only during the lifetime of a patent, which is usually 25 years, but also when the patent life of a drug is expired. As a result, the price of a drug when it becomes generic (*i.e.*, when there is no patent protection) remains about the same as when it was first introduced.

New patents are usually filed by the proprietor company on the same drug before the initial patent expires. The new patents may involve different drug formulations or related uses or different claims for the same or other diseases. This effort is usually undertaken by the initial patent holder company in order to safeguard the exclusivity of the monopoly, the high sale price and the level of the profits that can be made from the sale of their drug.

The sale price of a drug is another grey area affecting public health funding and drug availability in developed and developing countries. One of the contributory factors for the high price of new drugs is usually the inclusion of the marketing budget in the overall cost for drug development (Figure 1). For example, the costs for the organisation of conferences and the support for physicians to attend such conferences are included in the marketing budget and for fixing the price for the drug. Considering for example that the actual cost of producing and developing a new drug may be negligible, *e.g.*, less than 1 USD/g, the actual retail sale price may be in comparison greater than 1000 USD/g when the drug is under patent protection and produced by a multinational pharmaceutical company in a developed country. Similarly, if the same drug is not protected by a patent then the price could be less than 20 USD/g, if produced by a pharmaceutical company in a developing country. The same also applies when the patent expires and the drug becomes generic.

In general, the high price of drugs is a considerable obstacle in the provision of a better health care system in each country and diminishes the possibilities of supplying new improved drugs for the treatment of patients in developing countries and in some cases in the developed countries.

Many other controversial issues surrounding the development and use of new drugs include toxicity monitoring and differences between regulatory authority procedures among countries. Similar controversies involve the lack of transparency in the reporting of results of clinical trials, ineffective reporting of the adverse effects to the clinicians using the new drug by the pharmaceutical companies and the regulatory authorities, as well as misinformation on the risk/benefit assessment and criteria for the use of the new drug by comparison to generic drugs.

Although many pharmaceutical companies may be aware of the high toxicity of their newly introduced drug, they will continue selling it to acquire as much profit as possible. The new drug will not usually be withdrawn unless the company's profit margins are threatened or may be eliminated due to compensation claims or requests made by the affected patients and their legal representatives[20,100].

The drive for profit by multinational pharmaceutical companies and the lack of strict regulatory procedures or ethical codes endanger the safety of patients and the prospect of introducing optimum treatments. Furthermore, the present system adversely affects the economy of developing countries and health care resources of most developed countries. Within this context, it is highly unlikely that a pharmaceutical company would support any academic research for identifying and decreasing the toxic side effects of their drugs, following regulatory authority approval for clinical use and marketing. The same approach applies to research with generic drugs despite the fact that the manufacturing company involved may still have a sale monopoly or the drug may be tested for a different formulation or patent application.

Drug research, development and availability are relying at present almost exclusively on market forces and pharmaceutical companies' initiatives, marketing policies and decisions. This approach is not in many cases ethical and may not lead to optimal treatments and best patient care solutions. Similarly, the lack of health strategies and policies on drug design, development and use can overall influence patient treatment and safety[144].

There are no transparent procedures or specific ethical rules at present that will safeguard the rights of patients for the safest and most effective treatment for their disease or condition. The awareness and ethical approach for the treatment of each patient is the responsibility of the clinician in charge, who among others should provide the best care and prescribe the safest and most effective treatment and drugs. This responsibility is crucial for life threatening conditions, where the wrong risk/benefit assessment may result in ineffective treatments or serious toxicities and an overall increase in the morbidity and mortality rates. While organisations such as NICE in the United Kingdom may improve patient treatments and decrease costs, new organisations are needed to curb excess profits made by multinational pharmaceutical companies on new patented drugs. Such initiatives can decrease the cost of health care in developed countries and increase accessibility to drugs by patients in developing countries.

Improved measures on transparency regarding the reporting of clinical effectiveness and toxicity may help in the selection of the most appropriate and safe treatments for general, but also for individual patient use. Cost/effectiveness assessment issues are also important and may help orphan patients in developed as well developing countries[143].

Overall, it seems possible that with the appropriate health care policies, patient access to cheaper drugs can increase and waste on resources and health spending decrease. Such policies can benefit millions of orphan and other patients and may help in the treatment and elimination of many orphan diseases.

NEW OUTLOOK IN WORLD HEALTH ISSUES

Hunger, malnutrition, poor sanitation, impure water and lack of medicinal drugs appear to be the main causes of mortality and morbidity worldwide, affecting mostly children and infants in developing countries[28]. These problems can be overcome by increasing the production and supply of food, vaccines and medicinal drugs, as well as by improving water and sanitation technologies[1,2]. Within this context, a substantial reduction in the global mortality and morbidity levels can be achieved provided the appropriate health policies and strategies are implemented for each disease[25-27]. Such strategies should include further research on the mechanisms, drug treatment and prevention of diseases, population control through family planning, reduction of food waste in developed countries and reduction in environmental pollution.

Health resource allocation is a worldwide problem affecting all countries, health services and institutions and most categories of patients. Among these categories are orphan patients with rare, tropical and orphan diseases found both in developed and developing countries, who usually have limited access to treatments and also increased requirements for basic medications. The development of orphan drugs for such diseases can help many millions of patients worldwide. However, present conditions and policies are insufficient for overcoming these problems because of many limitations such as loopholes in regulatory and trade laws, misappropriation of public funds, bias in reporting and many other irregularities all of which undermine the efforts for improving the present status quo and benefiting affected patients (Figure 1)[20].

The present world trade and patent laws mostly benefit developed countries and affect the effective supply of sufficient food and drugs in the developing countries, where the resources are scarce and the appropriate technologies underdeveloped[12,14]. This can be illustrated by the sale of drugs amounting to about 0.5 trillion USD per year by the first twelve richest multinational pharmaceutical companies situated in the United States and Western Europe (Table 2). Such revenues are mainly generated from drugs sold for major common diseases. Interest

in drug development for neglected tropical diseases and rare, orphan diseases is limited, unless pharmaceutical companies are convinced that such projects can result in substantial profit returns[11]. The introduction of orphan drug legislation in developed countries has increased orphan drug development and global orphan drug sales which have steadily increased in the last few years and are now approaching 100 billion USD annually[13].

Market and monetary conditions appear to be the major determinant in government health policy, public health budgets and health resource allocation, which affect, to a great extent, the treatment of patients worldwide. However, it was recently realised by governments in many countries that allowing market forces and the free economy to influence and determine health conditions and resource allocation will be very costly and detrimental to the overall treatment and safety of patients[1,2,7,20].

Within this context, the institution of NICE in the United Kingdom and similar organisations elsewhere in developed countries is a limited step in the right direction, but does not address other major issues such as the pricing of drugs and the influence of pharmaceutical companies on governments, clinicians, pharmacists, regulatory authorities and other organisations. Similarly, it does not address many other problems such as pharmacoeconomic issues, literature bias and misinformation, transparency on drug efficacy and toxicity as well as development of generic drugs and nutraceuticals, and the availability of drugs at a lower cost than developing countries. One of the problems on drug selection and treatments is that NICE and similar organisations rely on published data, which are mostly biased due to the association with pharmaceutical companies and they are not generated by independent clinical investigators[5,19,20,24,50,141].

It appears that as the world population expands and the global economic situation is worsening, financial health resource allocation will become more important and related issues will be discussed on a wider platform in the state authorities, the medical literature and elsewhere. Similarly, initiatives may be taken by government bodies at different levels to decrease or limit the expenditure on drugs and medical devices, without lowering patient safety or treatment standards.

The paradigm of drug design and development of L1, which was mostly the result of academic initiatives and procedures, may prove to be a suitable model for the design of orphan drugs for orphan and rare diseases. This model has been shown to be more successful in comparison to the model of development used by pharmaceutical companies for DFRA, since L1 has been shown to be less toxic and more effective. Furthermore, as a result of transparent procedures a number of prophylactic measures were introduced as soon as the toxic side effects of L1 were identified and reported in the medical literature[17,127].

Transparency on the efficacy, toxicity and costs of the drugs is a major aspect of decision making for health policies and resource allocation. Despite the fact that such issues should be examined by expert public watchdog committees, most decisions rely on the pharmaceutical company's submission data and to a lesser extent on published data by independent investigators. Within this context, a relatively new science was developed, pharmacoeconomics, which is trying to address the cost of drugs and the impact on public health and society in general.

However, research reports suggest that there is evidence of publication bias with about 90% of pharmacoeconomics articles in most journals supporting the drug in question, compared to only 30% in the New England Journal of Medicine[52,141]. Similar articles sponsored by Novartis have been published for DFRA suggesting that DFRA is better value for money than DF, which however took into account much higher daily and weekly doses of DF, excluded the DFRA cost of toxicity monitoring and treatment outcomes as well as other relevant parameters[66,145]. Similar comparisons were also made by a different study sponsored by Apotex, one of the manufacturers of L1 in Western countries and by a company in Thailand, which showed much lower costs using L1 than other chelators[146,147].

Publication bias, misinformation, lack of transparency, selective reporting and other issues surround the publications sponsored by pharmaceutical companies, their academic consultants and medical writers[20,52,141]. Pharmaceutical company sponsored publications usually make excess claims on drug efficacy in contrast to toxicity, which is usually omitted or is scarce. Other marketing methods include misinformation reports on reduced efficacy, high toxicity and high cost of generic and other new competing drugs. Similar strategies, may involve the highlighting of reports of clinical studies using ineffective doses of generic and other new competing drugs or by making false claims on their toxicity.

Publication bias is also the responsibility of the editorial boards of journals, where the risk/benefit assessment for drugs is overtaken by other issues, which may ultimately influence patient treatments. In the case of L1 for example, an editorial dealt with a conflict between an academic and a pharmaceutical company (Apotex) without questioning the motives of the academic or the risk/benefit assessment on the treatment of the thalassaemia patients in developed and developing countries[76]. The same journal ignored its own earlier publications on the efficacy of L1 and its impact on the treatment of thalassaemia patients, probably because L1 was used under its chemical name and before the INN name Deferiprone was registered[69]. This conflict delayed the registration of L1 in the USA and Canada for more than twelve years, which may have resulted in many fatalities related to congestive cardiac failure, since L1 is known to effectively remove iron from the heart and reverse or prevent this form of iron toxicity[50,60,75,92].

There are many other examples of pharmaceutical company marketing policies for driving the market to adopt their product in addition to academic journals, such as the recruitment of patients in seeding trials, who eventually will remain on the treatment with the new drug after the trial, also in swinging opinion in support

of their new drug through conferences, through bribery and other methods (Figure 1)[130-133]. Such methods can influence patient treatments costing the health services billions of dollars, such as in the case of renal dialysis for end-stage renal diseases (ESRD). Emphasis on the prevention of progression of diabetic nephropathy *via* generic drug therapy may minimise such costs[148].

Despite the influence of pharmaceutical companies on public health resource allocation, recently there have been an increasing number of initiatives to change the status quo and overall improve public health care, including the prospect of orphan patient treatments in developing and developed countries. Examples include the successful lobbying by patient organisations for the introduction of L1 in the United States, the court ruling for cheaper imported drugs in India to help local patients and the clinical evaluation of EDTA by NIH in the United States for possible use in patients who suffered myocardial infarction and diabetes[55,149-151]. Further improvements can include the introduction of nutraceuticals, generic drugs and drug combinations in many orphan, rare and other diseases. For example, zinc has been suggested for use as adjunct treatment in infants with serious bacterial infections, which is one of the top causes of global mortality[152]. Similarly, the wider application of L1 and other generic drugs as main, alternative and adjuvant therapies in many conditions may result in more effective and less costly therapeutic options for many categories of orphan and other patients[55].

NEW POLICIES FOR IMPROVING DRUG AND HEALTH DEVELOPMENT

There are increasing prospects of reducing the rate of global mortality and morbidity by adopting specific policies that can have direct effects on many diseases including orphan, rare and tropical diseases. Improvement of food and health resources, better sanitation and drinking water purity, sex education and family planning are some of these policies which can play a major role in such efforts, especially in developing countries[1-3,153]. Similarly, the availability of drugs for the treatment of diseases is another major factor affecting the rate of global mortality and morbidity, especially patients in developing countries.

In contrast, different health policies can be introduced to reduce the rate of mortality and morbidity in the developed countries. Such policies can reduce, for example, environmental pollution, bad dietary habits, excess alcohol consumption and smoking which have been shown to lead to obesity, cancer, cardiovascular and other diseases. A similar policy is to tax such unhealthy habits similar to cigarettes and the relevant tax revenues to be used for subsidizing food supplies for malnourished people, vaccines, medicinal drugs and improve health education.

Health resource allocation can also be improved by reducing public health spending on drugs and diverting resources to other health areas which have an impact on the rate of global mortality and morbidity. This can be achieved through the adoption of policies that can limit excess profits made by multinational pharmaceutical companies and also through other measures that may facilitate the use of the best, safest and less costly treatments for the benefit of patients globally. Such measures and policies may include the sale price of drugs in developing countries to be adjusted as to the per capita ratio of developing to developed countries.

Transparency on the efficacy, toxicity and costs of manufacturing and sale of drugs including orphan drugs is essential for the development, application and safety of drugs in all diseases[66]. Within this context, the risk/benefit assessment of different drugs for each condition should be independently assessed and not rely only on published data, especially on studies sponsored by pharmaceutical companies. Identification of the therapeutic index of new and generic drugs and their impact on the treatment of each condition is also important for decreasing patient morbidity and mortality. Similarly, the implementation of drug safety measures is paramount for patient survival and well-being. In addition to the identification of toxic side effects, research on prophylactic measures and identification of drug antidotes can increase patient safety and survival.

The development of personalised medicine based on ADME, toxicity and other parameters is a further step in the achievement of improved therapeutic interventions[154]. Within this context, randomised clinical trials may not necessarily be the only tool of comparison for competing drugs in each condition. This is particularly important for generic drugs, where it may not be feasible to carry out such studies due to insufficient funding. The most important parameter for comparison in drug application in all diseases is whether the drug in question achieves a full treatment with acceptable level of toxicity. In cases where full treatment cannot be achieved, the effects on morbidity, mortality and cost of the drug under investigation should be compared to other drugs used for the same condition.

The paradigm of the development of L1 and other orphan drugs, which was based on academic and patient initiatives and efforts, appears to be a more successful, less costly and safer method in comparison to the monetary approach of pharmaceutical companies on orphan drugs. In such cases there are transparent procedures and scrutiny by academic peers with ethical instead of monetary motives. Similar academic initiatives involve the institution of drug combination treatments, which are studied and developed by academics independently of pharmaceutical companies and monetary motives[90-93,115]. Generic drug research and applications in other conditions such as the use of EDTA in many non-metal toxicity conditions by thousands of clinicians worldwide in millions of patients is another example of drug development independent of the pharmaceutical companies[55,64,150,151]. The development of other generic drugs and nutraceuticals is also increasing, especially in conditions where treatments are not successful or very expensive. Such approaches in-

clude the development and use of medicinal products in alternative and Chinese medicine.

The role of pharmaceutical companies is essential in manufacturing and developing drugs against diseases. This is a major contribution to society since it helps the treatment of billions of people every day. However, like any other business their main aim is profit and ethical considerations are secondary or absent in their agenda and policy planning. Within this context, their efforts are based on ways to maximise the income from the sale of their drugs. Unless government and regulatory authorities are vigilant and their medicinal drug policies are sufficiently effective, the treatment and safety of patients may be compromised by the activities of pharmaceutical companies including the exploitation of loopholes in regulatory and marketing procedures[20]. Similarly, the development of orphan drugs by pharmaceutical companies is not of major interest unless major financial benefits are clearly secured. Such policies and activities affect health resource allocation and especially orphan patients with orphan or rare diseases.

Drug selection and availability to patients in many cases resembles the sale of a market orientated product, like a chocolate brand or other food commodities. This can be observed for example in chelation therapy, where the drugs used for treatment are related to the marketing success of the manufacturing company and not the patient needs. The responsibility for allowing the manipulation of the drug market by pharmaceutical companies, which affects patient treatment outcomes and safety, lies exclusively with the government, the regulatory authorities and the clinicians. There are many recent examples of commercial company manipulations and government interventions, which may influence health resource allocation and outcomes. Such interventions include fines to settle civil and criminal investigations by the United States government in relation to sales and practices of various drugs by Pfizer totalling 2.3 billion USD, and a 3.0 billion USD fine for similar activities by Glaxo Smith Kline[155,156].

Several other measures and suggestions can also be considered in order to minimise the influence of pharmaceutical companies on patient treatment outcomes and safety and in reducing drug costs. For example, marketing expenses should not be included in the cost estimation and sale price of drugs. Similarly, the entry of pharmaceutical company marketing representatives should not be allowed in public hospitals for drug lobbying purposes, unless transparent procedures are followed and the time used for consultations is outside normal working hours. Manipulation of the drug market and prices and lack of transparency on safety, efficacy and costs should be penalized by the government authorities as shown in the United States, or where there are ineffective or corrupt regulatory authorities these can be replaced as shown, for example, in France[156,157].

The influence of pharmaceutical companies on academia including publications should be more transparent and the publication of studies sponsored by pharmaceutical companies should be cited as advertisements. Similarly, academics associated with pharmaceutical companies or the employees of pharmaceutical companies should not be used as referees or in editorial boards for drug related topics.

Members of public hospitals, local and international health authorities should be prevented from acting as consultants for private pharmaceutical companies unless such activities are through transparent procedures and commercial involvement is outside public service responsibilities. The support received by clinicians from pharmaceutical companies including participation in conferences and clinical trials should also be through transparent procedures. Similarly, results from clinical trials on drugs should be independently reported and not controlled by medical writers and others belonging to the proprietor pharmaceutical company. Public expenditure in relation to doctors' absences for conferences organised or sponsored by pharmaceutical companies should not be considered as further education activities, unless these are organised by independent academic societies or non-profit academic organisations.

These and many other policies and measures may help to decrease the expenditure by health authorities on drugs both in developed and developing countries. Similarly, it may also help in the adoption of more transparent procedures and the improved allocation of health resources which may lead to better patient treatment outcomes and better safety. In the meantime, many research challenges are continuously emerging in many orphan, rare and other diseases which may influence health outcomes, morbidity and mortality as shown by L1 and other drugs[158-162].

The world efforts for better redistribution of global wealth may be the answer to health resource allocation, since the major cause of mortality at present is associated with poverty, malnutrition and starvation. Present estimates suggest that 1% of the world population owns 50% of the global wealth and that more than 3.5 trillion USD are deposited in tax heavens by rich individuals and commercial companies.

CONCLUSION

World health developments including morbidity and mortality outcomes are a reflection of many factors which are affected by health policies in individual countries and globally. Food availability, health provision and education, family planning, disease prevention, nutrition, environmental and monetary influences, genomic and psychological aspects are some of the factors which are in dynamic equilibrium and can influence health levels and outcomes in each country. There is scope for substantial improvements in world health policies and many ethical dilemmas and issues related to health strategies need to be prioritised, readdressed and resolved in each country and also globally. The disease profile and health policies between developed and developing countries are different, with profound financial resource insufficiencies in the latter.

The availability and cost of generic and new medicinal drugs are among the major areas affecting the level of global health care. Monetary, ethical and other issues affect the supply of medicinal drugs for different categories of patients in each country. Health policies, regulatory and marketing procedures can variably influence the risk/benefit assessment, patient safety, drug availability and drug treatment outcomes in each country. Public health and overall national spending are also influenced by such procedures. Reassessment of drug pricing and of regulatory procedures with major emphasis on the development of orphan drugs based on a risk/benefit assessment may help in the treatment of many categories of orphan and rare diseases and millions of orphan patients globally. The criteria for drug development and use and of price levels in each condition should be readdressed and modified to improve patient treatments, drug safety and minimise costs.

The implementation of improved policies on health resource allocation and drug development can lead to the realisation of many major health aims such as the introduction of worldwide and universal health care. Similarly, advances in medical research can lead to the elimination and improved treatment of many diseases, to an overall reduction in the morbidity and mortality rates and an increase in the quality of life for patients worldwide.

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MINIREVIEWS

Dyspepsia and celiac disease: Prevalence, diagnostic tools and therapy

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Abstract

The prevalence of dyspepsia is up to 40% in population-based study. Functional dyspepsia is an exclusion diagnosis and it is classified as a chronic abdominal pain-related functional disorder, characterized by the presence of persistent or recurrent pain or discomfort centered in the upper abdomen, neither relief by defecation, nor association with the onset of a change in stool frequency or form. Celiac disease (CD) is a common autoimmune enteropathy, with a prevalence around 1% in the general population. Its diagnosis includes a serological screening and an upper gastrointestinal endoscopy with multiple biopsies. Gluten-free diet is the only effective treatment. CD diagnosis is often delayed in asymptomatic patients or in individuals with less clinical gastrointestinal symptoms. Several studies performed coeliac disease screening in patients with symptoms suggestive of dyspepsia, showing a biopsy-proved prevalence that ranged from 0.5% to 2%. The typical endoscopic markers of villous atrophy are not sufficiently sensitive, so some endoscopic techniques, such as "water immersion" and confocal endomicroscopy were proposed to improve the diagnostic sensitivity and target biopsies. A recent meta-analysis estimated that the prevalence of CD was higher in patients with dyspepsia, but not in a statistically significant way. However this assumption should be confirmed further larger studies.

Key words: Dyspepsia; Coeliac disease; Upper endoscopy; Villus atrophy; Screening

Core tip: Dyspepsia is classified as a chronic abdominal pain-related functional disorder that affects almost 40% of the population. It can be also a manifestation of celiac disease, an immuno-mediated enteropathy, caused by the ingestion of gluten in genetically predisposed patients. The prevalence of celiac disease among dyspeptic patients has been investigated, with results ranging from 0.5% to 2%. Celiac disease diagnosis requires histological evaluation of villous atrophy on duodenal biopsies specimens. Screening for celiac disease in dyspeptic patients and routinely performing of biopsies during upper gastrointestinal endoscopy, may be useful as part of the diagnostic flow-chart of these patients.

Petrarca L, Nenna R, Mastrogiorgio G, Florio M, Brighi M, Pontone S. Dyspepsia and celiac disease: Prevalence, diagnostic tools and therapy. *World J Methodol* 2014; 4(3): 189-196 Available from: URL: http://www.wjgnet.com/2222-0682/full/v4/i3/189.htm DOI: http://dx.doi.org/10.5662/wjm.v4.i3.189

INTRODUCTION

Dyspepsia is one of the most common gastrointestinal disorders to be faced in clinical practice, with prevalence up to 40% in population-based study[1] so that the economic impact is very high.

When dyspepsia is not a manifestation of an organic

pathology, such as gastroesophageal reflux disease or peptic ulcer disease, then it is classified as functional dyspepsia (FD).

FD markedly reduces patients' quality of life, similarly to mild heart failure and menopause[2]. However FD is an exclusion diagnosis and on the basis of Rome Ⅲ criteria[3], it is defined as the presence of gastroduodenal symptom without evidence of structural disease able to explain the syptoms. Often patients refer to suffer from early satiation or postprandial fullness (postprandial distress syndrome), epigastric pain/discomfort or burning (epigastric pain syndrome).

Pathophysiology of FD is not completely understood yet and several pathophysiological mechanisms have been proposed to underlie symptoms. Central processing of visceral stimuli, low-grade inflammation in the duodenum and genetic factors are the main emerging hypothesis investigated[4]. FD is difficult to manage, because no medication is currently approved in the United States, Canada or the European Union. Many treatments have been proposed (diet, eradication of *H. pylori* and drugs such as prokinetic agents or protonic pump inhibitors)[5] but no one was satisfactory.

Celiac disease (CD) is an auto-immune enteropathy, whose diagnosis is often delayed in asymptomatic patients or in individuals with less clinical gastrointestinal symptoms, such as abdominal bloating, nausea and vomiting. CD diagnosis, according to the American Gastroenterology Association, consists of a serological screening (including anti-transglutaminase, anti-endomisium and anti-deamidated gliadin antibodies) and an upper gastrointestinal endoscopy with multiple duodenal biopsies. Gluten-free diet is the only effective treatment for the disease.

However, although dyspepsia may be a manifestation CD, most of FD patients do not perform serological screening for CD or duodenal biopsies and there are few data about the prevalence of CD in patients with dyspepsia.

Recent studies[6-10] demonstrates that the prevalence of silent CD in patients with dyspepsia is slightly higher than that of the general population, however in one study it resulted rather low[11].

The 40%-60% of subjects with dyspepsia resulted macroscopically normal when performing upper gastrointestinal endoscopy[12]. Unfortunately, the practice of performing biopsies, even in absence of endoscopic alteration of intestinal mucosa, is quite uncommon.

The typical endoscopic markers of villous atrophy include mosaic pattern, scalloping of folds, and a decrease of duodenal folds. However, mostly in less severe cases, CD diagnosis cannot only be performed on these parameters. So, considering that many authors describe these markers as not sufficiently sensitive, some endoscopic techniques, such as "water immersion" and confocal endomicroscopy (CEM) were proposed to improve the diagnostic sensitivity and target biopsies in most damaged mucosal areas[13,14].

DYSPEPSIA AND CELIAC DISEASE

Recent studies demonstrate that the prevalence of CD in patients with dyspepsia is higher than that of the general population[6-10].

Bardella *et al*[6] prospectively enrolled 517 patients suffering from dyspeptic symptoms. All patients were submitted to upper gastrointestinal endoscopy, and six were diagnosed to be celiac (1.2%). Interestingly three patients (50%) had a normal duodenal endoscopic pattern and five of the six celiac patients were young women aged between 20 to 37 years. The authors suggest to perform serological screening for celiac disease especially in young women suffering from dyspepsia.

Lima *et al*[7] reported a CD prevalence of 1.4% in a small series of patients with dyspepsia, both were young women, aged 19 and 25 years respectively. In the paper of Ozaslan *et al*[8] among the 196 investigated patients three were diagnosed to be celiac (1.5%). All were female younger than 52 years, and only two showed abnormal endoscopic findings.

In the manuscript by Giangreco *et al*[9], published in 2008, the role of upper gastrointestinal endoscopy in CD diagnosis was evaluated in patients suffering from FD. The prevalence of CD was 2% (15 patients out of 726 enrolled), higher than the general population one, also considering that patients with an increased risk for CD (such as first degree relatives) were excluded from the study. Among the 15 CD patients (age ranged 20 to 56): 10 were female and only 8 patients presented endoscopic findings suggestive for CD.

Keshavarz *et al*[10] investigated the prevalence of CD among 170 patients with FD. Twelve patients (10 female), suffering form dysmotility-type dyspepsia, tested positive for CD related antibodies, however only two of them showed villous atrophy at the histological evaluation.

Only in the paper by Heikkinen *et al*[11] published in 1995, among the 400 uselected dyspeptic patients enrolled to perform upper gastrointestinal endoscopy, serological evaluation and abdominal ultrasound, CD was diagnosed in 2 patients (both aged less than 64 years). The low prevalence (0.5%) could be due, maybe, to the eterogeneity of the population study, with a higher percentage of aged patients (77% were more than 44 years old) while the most frequent diagnosis in younger patients was lactose intolerance (9%).

In a recent meta-analysis by Ford *et al*[15], the authors provided a pooled prevalence of biopsy-proven CD of 1.0%, similar to that in the general population, when duodenum biopsy was performed as first-line investigation. However when the authors pooled the data from the studies that used the Rome Ⅱ criteria for dyspepsia, the biopsy proved CD was 2%, significantly higher.

CD

CD is a chronic, immuno-mediated enteropathy, caused by ingestion of gluten in susceptible individuals, carrying DQ2 and/or DQ8 HLA. It is characterised by a chronic inflammatory state of the small intestine that recover after gluten withdrawal. The typical changes of the duodenal mucosa include: raised intra-epithelial lymphocyte, crypt hyperplasia and various degree of villous atrophy

as classified by Marsh and modified by Oberhuber *et al*[16] in 1999, that decreased digestion of food and micro- and macronutrients absorption.

CD is common, with a prevalence around 1% in the general population of Western countries[17,18], more frequent in females than males.

Pathogenesis

The pathogenesis is multifactorial, including the interactions between environmental, genetic and immune factors. Gluten, a protein derived from wheat, barley and rye, represents the trigger factor of CD. The alcohol-soluble fraction of gluten, the alpha-gliadin, is rich in prolamine and glutenine that could trigger an immune response, mediated by both innate and adaptative arms of CD patients' mucosal immune system. Genetic susceptibility plays a crucial role in CD pathogenesis, as demonstrated by the increased prevalence in first-degree relatives (9.5%) and siblings (11%)[19]; in the homozygous twins it arises to 75%[20,21]. The genetic basis of celiac disease can be divided between *HLA* and *non-HLA* gene variants[22].

The HLA DQ2 heterodimer is present in 90% of celiac patients, in 5% of the cases the HLA DQ8 heterodimer is present. The HLA DQ2 heterodimer, present in 90% of celiac patients[23], is formed by a beta chain (β) encoded by the allele HLA DQB1 * 02 (HLA DQB1 * 0201 or * 0202) and by an alpha chain (α) encoded by the allele HLA DQA1 * 05. The heterodimer HLA DQ8 is formed by a β chain and an α chain encoded by HLA DQB1 * 0302 and HLA DQA1 * 03 respectively[24].

Genes of the HLA complex can contribute in only 36% of the increased risk of celiac disease in siblings[22], indicating the need for assistance from other *non-HLA* genes[25].

The frequent association of celiac disease with other monogenic diseases may demonstrate the existence of a link with other genes on chromosome 7 (short arm) implicated in Williams syndrome and on chromosome 21 involved in Down syndrome[26].

A fundamental role in the pathogenesis is carried out by an ubiquitous calcium-dependent enzyme, the transglutaminase type 2 (TG2). The TG2 catalyzes the acyl transfer between the γ-carboxamide group of glutamine and the ε-amino group of lysine or primary amine soluble. This mechanism forms gliadin-gliadin macromolecular complexes, which are considered neoepitopes, therefore non-self antigens against which the immune system reacts.

In the presence of a low pH, an abundance of glutamminic residues and scarcity of proteins that bind lysine, TG2 catalyses the deamidation of glutamine[27-29]. Some of these peptides of "deamidated" gluten, because of their negative charge, show a high affinity for the HLA-DQ2 or-DQ8 heterodimer. Once bound to these molecules they activate intestinal mucosa T cells[30-32] and they cause the cytokine production and the begins of the intestinal damage.

Clinical presentation

The first modern description of CD is due to Samuel Gee, an English paediatrician, published in the St. Bartholomew's Hospital Reports of 1888. He recognised CD as a chronic indigestion, occurring in people of all ages, presenting as diarrhoea.

Nowadays clinical presentations of CD may vary from silent to severe malabsorption symptoms (celiac crisis).

Didactically, CD manifestations are divided in: (1) typical: including gastrointestinal symptoms, such as diarrhoea, weight loss, abdominal pain, failure to thrive, abdominal distension and vomiting; (2) atypical: that is for example short stature, iron-deficiency anaemia, dermatitis herpetiformis, delayed puberty; and (3) silent: completely asymptomatic.

A delayed gastric empty and a slow oro-caecal transit has been observed in celiac patients on a gluten containing diet, probably due to abnormal exposure of small bowel unabsorbed starch and fats and to altered neuroimmunomodulation and hormonal deregulation (low levels of cholecystokinin and high levels of peptide YY)[33]. Some authors investigated the transit disorders in patients with untreated CD using the video-capsule endoscopy. Urgesi *et al*[34] found that there was no difference in gastric empting and small bowel transit time between CD patients and control group. However, Ciaccio *et al*[35] observed changes in motility of the small bowel and they speculated that the reduced folds can cause more rapid changes in the position and in the width of the luminal centre.

Diagnosis

CD diagnosis, according to the American Gastroenterology Association, consists of a serological screening and an upper gastrointestinal endoscopy[36].

Nowadays, CD serological screening is recommended for symptomatic patients, or for those people who are at high risk of CD (such as first degree relatives). It encompasses the total serum IgA, the IgA anti-transglutaminase antibodies (AbTG2), IgA anti-endomisium antibodies (EMA) and IgA anti-deamidated gliadin antibodies (DGP).

AbTG2 proved to have a very high sensitivity (98%-100%) and a very good specificity (94%-98%)[37], they are the most widely used for CD screening, even if they can be found in patients affected by other autoimmune diseases[38]. They can be determined both by ELISA or RIA, the latter technique showing a so high sensitivity[39] that it has been used to detect AbTG2 in saliva[40], demonstrating a correlation with CD histological grading and diffusion of duodenal lesions[41].

EMA have a very high specificity (100%), but a lower sensitivity than AbTG2[38]. They are determined by indirect immunofluorescence, using monkey oesophagus sections as substrate.

DGP have been demonstrated to be more sensitive and specific than the old antigliadin antibodies and they are useful especially in children younger than two years

of age[42].

In IgA deficient patients, it is recommended to perform the IgG antibodies, particularly IgG anti-deamidated gliadin antibodies.

The upper gastrointestinal endoscopy with multiple biopsies, both from duodenal bulb and distal duodenum, is the gold standard for diagnosis[36]. The standard endoscopy does not permit the visualization of villous atrophy, even if several macroscopic markers has been related to CD (Figure 1), such as reduction or absence of duodenal folds, scalloping, nodular appearance and mosaic pattern. However the power of these endoscopic markers to predict the villous atrophy is still debated[43,44]. This variability could be due to the absence of macroscopic sign in case of patchy or partial villous atrophy.

In the last few years, new methods have been developed to evaluate with more accuracy the macroscopic appearance of villous pattern during upper gastrointestinal endoscopy.

The water immersion technique (Figure 1C) is an easy procedure that can emphasize the villous pattern. It consist in a first phase of air suction from the lumen, then a second phase of injection of 90-150 mL of water[45]. It has the potential to target biopsies and, eventually, reduce the number of specimen, thanks to its capability of enhancing areas of villous atrophy. An alternative technique is represented by the chromoendoscopy, that uses the dye staining with indigo carmine in enhancing the visualization of the mucosal surface. This endoscopic tool has showed a better accuracy when combined with magnification endoscopy[14].

Narrow band imaging (NBI) is another technology that improves the visualization of the surface of the superficial mucosa and its vascular architecture.

NBI with optical magnification assists in detecting patients with villous atrophy without determining the level of intraepithelial lymphocytosis and crypt hyperplasia[46,47].

Cammarota *et al*[48] reported their experience in the use of I-scan technology during endoscopy for the evaluation of the duodenal villous pattern. It works in real time and permits to switch from standard endoscopy to I-scan view very quickly. The authors reported an accuracy of 100% in detecting total villous atrophy, and suggested a possible role of this technique in targeting biopsies in patchy distribution of lesions. However in the reported study, all the enrolled patients underwent upper gastrointestinal endoscopy for suspicion of malabsorption, so they had a high pre-test probability of duodenal atrophy.

Rokkas *et al*[49] recently published a meta-analysis about the role of video capsule endoscopy in CD diagnosis and reported a pooled sensitivity of the tool of 89%. A normal capsule endoscopy cannot exclude CD, however it could provide information on the extent of the disease, allowing the visualization of not accessible portion of small bowel, even thou the histological evaluation of bioptic samples still remain the gold standard for the diagnosis.

Biopsies taken during endoscopy must be oriented on filter paper, fixed in formalin and embedded in paraffine. After the cut and haematoxylin-eosin staining, an expert pathologist assesses the sections under light microscopy, evaluating the intraepithelial lymphocytes (IEL) count, the villo/crypta ratio and the villous atrophy using the Marsh modified by Oberhuber classification[16]: (1) type 0: normal mucosa with less than 40 IEL/100 enterocites (EC); (2) type 1: infiltrative, that is characterised by normal villous architecture, normal crypt height, but high IEL counts (> 40/100 EC); (3) type 2: hyperplastic, with a normal villous architecture, but but high IEL counts (> 40/100 EC) and crypt hyperplasia; (4) type 3: destructive, in which besides a high IEL counts (> 40/100 EC) and acrypt hyperplasia, it can be also observed a villous atrophy (3a: mild villous atrophy, 3b moderate villous atrophy, 3c: total villous atrophy).

Recently a new classification has ben proposed by Corazza *et al*[50], in order to reduce the inter and itra-observer disagreements and to facilitate the relationship between pathologists and gastroenterologist. It consists of two degrees: (1) A: non-atrophic lesions of the duodenum; and (2) B: atrophic lesions. It is divided into grade B1, that include mild and moderate villous atrophy, and grade B2, with a total villous atrophy.

The intestinal involvement, however, is not always confined to the duodenum. It has been demonstrated that other portions of the gastrointestinal tract are involved, such as the gastric[51], oral[52] and colonic mucosa[53].

The chronic superficial gastritis has been described as the most frequent form of gastritis that occurs in non treated celiac patients[54], followed by lymphocytic gastritis, a form of gastritis of uncertain pathogenesis[55].

Therapy

Gluten-free diet is, at this moment, the only effective treatment for coeliac disease, allowing the healing of intestinal mucosa, the improvement of symptoms and prevents the onset of long-term complications, such as osteoporosis[56] and autoimmune disorders[57,58].

However, many efforts have been made to find an alternative therapy for CD, involving the biotechnology field, which led to a better understanding of the molecular mechanisms of coeliac disease and the identification of pathogenetic pathways that could be targeted by new drugs. Currently the main targets under investigation are[27]: (1) endopeptidases capable to detoxify gluten in order to decrease its immunogenic power; (2) modulation of permeability by the pill AT-1001; (3) block of antigen presentation made by inhibitors of TG2 and HLA-DQ2; (4) inflammation modulation using monoclonal antibodies directed against inflammatory cytokines; (5) block of the recruitment of lymphocytes by molecules that inhibit the migration to the intestinal mucosa; and (6) immunomodulation and induction of gluten tolerance.

In the last few years a new gluten- related syndrome is increasing awareness: the non celiac gluten sensitivity (NCGS). NCGS often overlaps with irritable bowel disease syndrome and for both conditions the diagnosis is

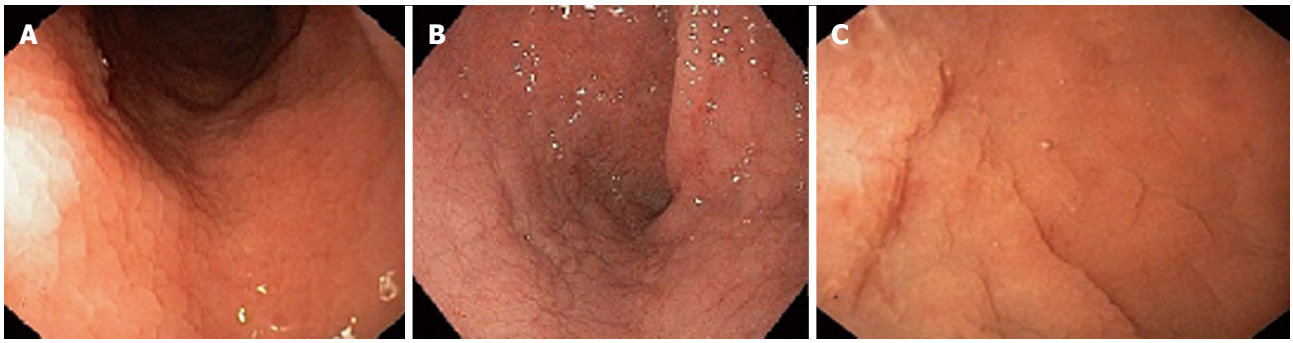

Figure 1 Endoscopic markers of celiac disease. A, B: The typical endoscopic markers of villous atrophy include mosaic pattern, scalloping of folds, and a decrease of duodenal folds; C: Appearance of the duodenum of a celiac patient using the water immersion technique.

based on clinical symptoms.

However it is a still poorly defined syndrome, characterized by the presence of gastrointestinal symptoms, such as bloating, abdominal pain, nausea, gastroesophageal reflux disease, and/or extraintestinal manifestation, tiredness, headache, anxiety, foggy mind and peripheral numbness[59]. The clinical presentation of these symptoms has been associates to the ingestion of gluten, however it has been hypothesize that other wheat proteins, such as amylase trypsin inhibitors, could play a role[60]. Also fermentable oligosaccharides, monosaccharides and disaccharides, contained in wheat, rye, but also in milk, legumes, honey and some vegetables (fennel, beetroot, and chicory) has been proposed to be important in NCGS[61]. NCGS is an exclusion diagnosis, so CD and wheat allergy should be rouled out. Its prevalence is still uncertain, ranging from 3.19%[59] in Italy, to 6% in United States[62] and it is more frequent in female than in male. Further study, including possibly a double blind gluten challenge, should be performed to assess the real prevalence of NCGS.

CONCLUSION

CD diagnosis is often delayed in asymptomatic patients or in individuals with less clinical gastrointestinal symptoms, such as abdominal bloating, nausea and vomiting, despite the many benefits deriving from a prompt identification.

Based on these assumptions, several studies performed coeliac disease screening in patients with symptoms suggestive of dyspepsia, showing a biopsy-proved prevalence that ranged from 0.5% to 2%[6-11]. Interestingly, the subgroup of dyspeptic patients at highest risk comprised young women, aged from 20 to 37 years (RR of CD 3.22)[6].

The 40%-60% of subjects with dyspepsia resulted macroscopically normal when performing upper gastrointestinal endoscopy[63,64]. Unfortunately, the practice of performing biopsies, even in absence of endoscopic alteration of intestinal mucosa, is quite uncommon.

The typical endoscopic markers of villous atrophy include mosaic pattern, scalloping of folds, and a decrease of duodenal folds (Figure 1). However, mostly in less severe cases, CD diagnosis cannot only be performed on these parameters. So, considering that many authors describe these markers as not sufficiently sensitive, some endoscopic techniques, such as "water immersion" and CEM were proposed to improve the diagnostic sensitivity and target biopsies in most damaged mucosal areas[12-14].

A recent meta-analysis by Ford *et al*[15] evaluated the yield of diagnostic testing for CD in patients affected by dyspepsia. The pooled prevalence of positive celiac serology ranged from 6% to 8%. The author pooled the data from literature and estimated that the prevalence of positive celiac serology ranged from 6% to 8%, the biopsy-proved CD prevalence was also higher in patients with dyspepsia, approximately 2%, than controls, but not in a statistically significant way. However, due to several limits that affected the paper, such as presence of study based only in tertiary care, this assumption should be confirmed further larger and, possibly, case-control studies.

In conclusion screening for CD in patients suffering from dyspeptic symptoms, as defined by Rome Ⅲ criteria, and routinely performing of biopsies during upper GI endoscopy, may be useful as part of the diagnostic flow-chart of these patients, considering the benefits of a promptly beginning of a gluten-free diet, even thou further, well-defined and case-control studies on a larger population could definitively assess if CD prevalence is higher in dyspeptic patients.

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REVIEW

Transition of Thalassaemia and Friedreich ataxia from fatal to chronic diseases

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Author contributions: Kolnagou A reviewed the organisational health structure of thalassaemia and Friedreich ataxia in Cyprus; Kontoghiorghe CN contributed the literature background on recent developments on thalassaemia and Friedreich ataxia and critically reviewed the clinical and other aspects of the manuscript; Kontoghiorghes GJ designed, wrote and edited the manuscript including the mechanisms of iron chelation therapy and iron metabolism and toxicity.
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Abstract

Thalassaemia major (TM) and Friedreich's ataxia (FA) are autosomal recessive inherited diseases related to the proteins haemoglobin and frataxin respectively. In both diseases abnormalities in iron metabolism is the main cause of iron toxicity leading to increased morbidity and mortality. Major efforts are directed towards the prevention of these diseases and also in their treatment using iron chelation therapy. Both TM and FA are endemic in Cyprus, where the frequency per total population of asymptomatic heterozygote carriers and patients is the highest worldwide. Cyprus has been a pioneering nation in preventing and nearly eliminating the birth of TM and FA patients by introducing an organized health structure, including prenatal and antenatal diagnosis. Effective iron chelation therapy, improved diagnostic methods and transfusion techniques as well as supportive therapy from other clinical specializations have improved the survival and quality of life of TM patients. Despite the tiresome clinical management regimes many TM patients are successful in their professional lives, have families with children and some are now living well into their fifties. The introduction of deferiprone led to the elimination of cardiac failure induced by iron overload toxicity, which was the major cause of mortality in TM. Effective combinations of deferiprone with deferoxamine in TM patients caused the fall of body iron to normal physiological ranges. In FA different mechanisms of iron metabolism and toxicity apply to that of TM, which can be targeted with specific iron chelation protocols. Preliminary findings from the introduction of deferiprone in FA patients have increased the hopes for improved and effective therapy in this untreatable condition. New and personalised treatments are proposed in TM and FA. Overall, advances in treatments and in particular of chelation therapy using deferiprone are transforming TM and FA from fatal to chronic conditions. The paradigm of Cyprus in the prevention and treatment of TM can be used for application worldwide.

Key words: Thalassaemia; Friedreich ataxia; Prenatal diagnosis; Survival; Chelation therapy; Deferiprone; Deferoxamine; Cyprus

Core tip: Thalassaemia major (TM) and Friedreich's ataxia (FA) are inherited diseases related to iron toxicity, with high morbidity and mortality rates. Cyprus has the highest frequency of TM and FA worldwide. Prenatal diagnosis and other health policies almost abolished the birth of TM and FA patients in Cyprus. Deferiprone has increased the survival and quality of life of TM patients, who are now reaching normal life expectancy and it is also promising for FA patients. Personalised treatments are proposed for TM and FA. The Cyprus experience can be used as a paradigm for the prevention and treatment of TM worldwide.

Kolnagou A, Kontoghiorghe CN, Kontoghiorghes GJ. Transition of Thalassaemia and Friedreich ataxia from fatal to chronic diseases. *World J Methodol* 2014; 4(4): 197-218 Available from: URL: http://www.wjgnet.com/2222-0682/full/v4/i4/197.htm DOI: http://dx.doi.org/10.5662/wjm.v4.i4.197

INTRODUCTION

Thalassaemia major (TM) and Friedreich's ataxia (FA) are autosomal recessive inherited diseases with serious pathological complications, morbidity and mortality. Although the two diseases are genetically different they are both related to abnormalities in proteins of iron metabolism namely frataxin in FA and haemoglobin in TM[1-5].

Adult haemoglobin is composed of two alpha and two beta globin chains, each containing an iron molecule embedded in a protoporphyrin ring, which is responsible for the transport of oxygen to all cells of the body[1]. Frataxin is a mitochondrial matrix protein which functions in iron-sulfur cluster containing enzymes within the chain assembly responsible for respiration and energy transduction[3,4].

While frataxin is encoded by the gene of chromosome 9, the beta globin chains of haemoglobin are encoded by a single gene on chromosome 11 and the alpha globin chains of haemoglobin are encoded by two genes which are closely linked on chromosome 16[1,3,4].

In patients with TM, insufficient or no beta globin chains of haemoglobin are produced and the abnormal haemoglobin cannot deliver oxygen efficiently to the tissues. TM is a fatal disease if it is not treated with regular blood transfusions every 1-4 wk and chelation therapy[5].

FA is a progressive neurodegenerative disease with significant morbidity and has no effective treatment[6]. In FA patients the production of frataxin is severely reduced. Frataxin is a highly conserved mitochondrial matrix protein composed of 130 amino acids, has MWt 14.2 kDa and weakly binds iron[3,4]. In almost all FA patients there is an expansion of the guanine-adenine-adenine (GAA) trinucleotide in the first intron of both alleles of the frataxin gene. While in normal individuals the alleles of the frataxin gene have 36 or fewer GAA repeats, in FA disease the alleles have approximately 70 to more than 1200 to 1700 GAA repeats[3,4,6].

Major efforts have been taken worldwide for the control and reduction of births of TM and FA patients. Prenatal and antenatal diagnoses are increasingly being used in certain endemic areas and in ethnic groups for the prevention of these and other inherited diseases. Cyprus has the highest rate of asymptomatic heterozygote carriers and patients per population of both TM and FA by comparison to any other nation[7-11]. There is a 25% chance of TM or FA child being born from a pairing of asymptomatic heterozygote carrier parents. Within this context an effective prevention programme is needed for the containment of TM and FA in endemic areas like Cyprus.

Regarding patient treatment, recent developments involving primarily iron chelation therapy using the iron chelating drug deferiprone (L1) suggest that major advances have taken place in reducing morbidity and mortality in TM and also promising steps towards improvement in the treatment of FA[5,12]. The strategy responsible for these advances and the transition of these diseases from the fatal to the chronic stage can better be described using the paradigm of Cyprus[9]. Within this context the recent developments in the past few years in TM and FA in Cyprus have been reviewed with particular emphasis on the strategies regarding the health organisation structures, educational efforts, prevention measures and the overall clinical management. Related epidemiological and socioeconomic consequences are also described.

Special emphasis is also given to the mechanisms and pathogenesis of gross or focal iron overload toxicity in both TM and FA[5]. The treatment of gross or focal iron overload toxicity using chelation appears to be the major therapeutic option for decreasing the associated high mortality and morbidity observed in these two categories of iron metabolic diseases. The role of L1 is highlighted, since it is considered as one of the major factors in the transition of TM from a fatal disease to a chronic disease and also increases the prospects of effective treatment in FA[5].

EPIDEMIOLOGY, TREATMENT AND SURVIVAL OF THALASSAEMIA PATIENTS

TM is classified as a haemoglobinopathy, the most common group of inherited diseases in humans. More than 200 mutations of the haemoglobin genes have been reported, causing a range of pathological abnormalities from asymptomatic to fatal states[1,2]. Patients with TM can only survive if they are regularly transfused with normal red blood cells from appropriate blood donors. Iron accumulated from regular long term red blood cell transfusions is toxic to many organs and becomes fatal unless removed by chelating drugs. Iron overload toxicity in TM has one of the highest metal related morbidity and mortality rates globally[13].

The geographic distribution and prevalence of thalassaemia is mostly in developing countries found in the Mediterranean, Middle East and South East Asia, where over 90% of thalassaemia patients are born. More than 100000 thalassaemia babies is estimated to be born annually and there are 100 millions of thalassaemia heterozygote asymptomatic carriers worldwide[2]. In India alone the annual birth rate of TM is estimated at 9000[14].

Thalassaemia is considered as an orphan disease in the European Union (EU), the United States and other developed countries due to the low number of patients compared to the total population, which is mainly Caucasian[2,15-17]. The treatment of TM patients in EU countries (*e.g.*, Cyprus, United Kingdom, Greece and Italy) is supported by the state, while in most developing countries insufficient funds are available for their treatment.

Organisation structure and collaborating bodies involved with the coordination for the prevention diagnosis and treatment of thalassaemia in Cyprus

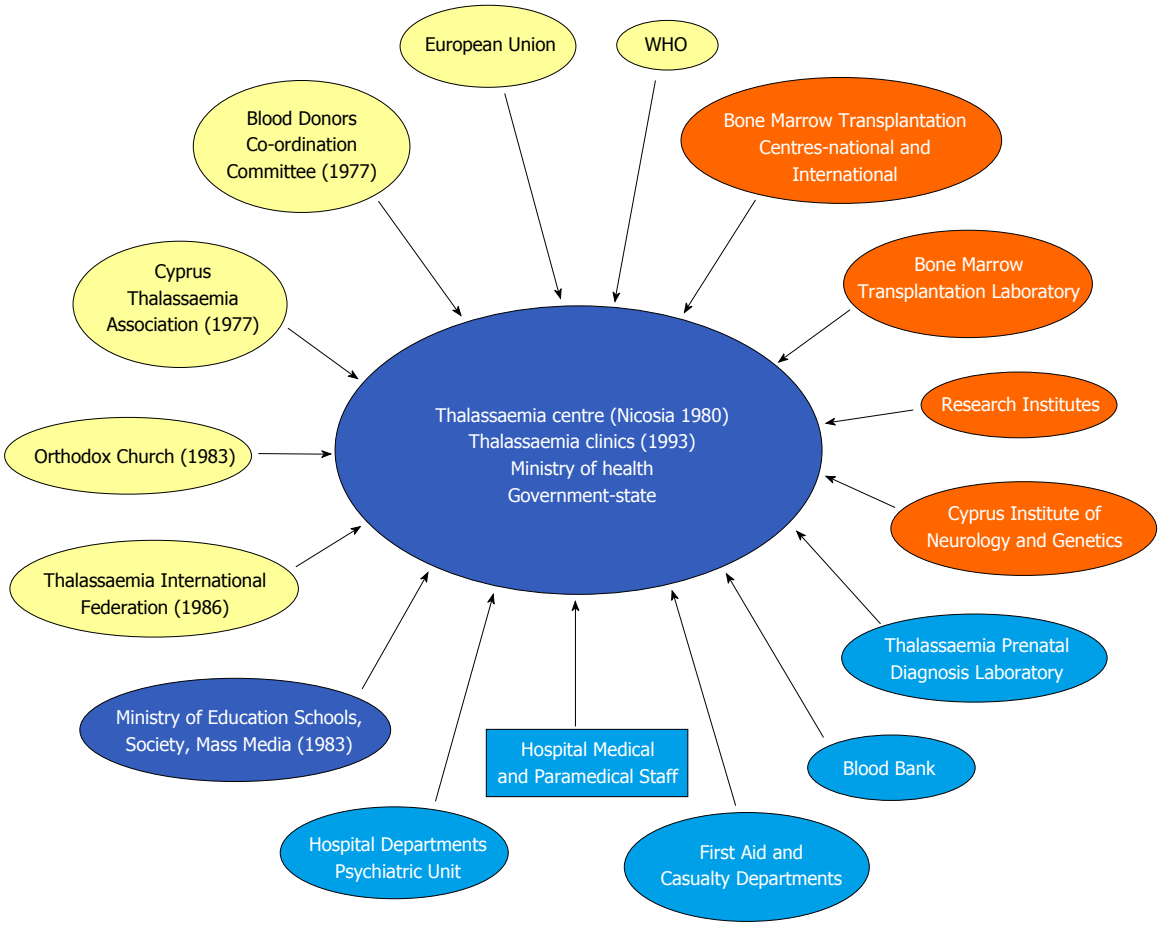

Figure 1 The organisational health structure and the chronological development of Thalassaemia in Cyprus. WHO: World Health Organization.

In the case of Cyprus, epidemiological data suggest that the indigenous population is numbered at around 800000 thousand and an extra 200000 thousand are mostly immigrants from developing countries working in Cyprus. The thalassaemia heterozygote frequency, which is associated mainly with the beta globin chain trait is 1 in 6 Cypriots. There are about 800 TM and thalassaemia intermedia patients ie (1 in 1000) who are regularly transfused and treated in specialist thalassaemia clinics in the main cities of Cyprus (Nicosia, Limassol, Larnaca, Paphos)[9].

Historically, the introduction of government policies to control thalassaemia came about after Cyprus gained independence in 1960. Before this period TM patients in Cyprus had a similar fate to TM patients in developing counties, where there is inadequate treatment including blood transfusions and chelation therapy[14,18,19].

Estimates from the health budgets for the treatment of TM patients were colossal for the size of Cyprus at the time of independence. Projections for the rate of birth of TM children and the associated expenditure on treatment predicted the collapse of the national health service or abandonment of the treatment due to unaffordable costs. The socioeconomic consequences for the families with TM children were devastating.

Based on these dilemmas the government of Cyprus introduced a new policy about 35 years ago for the set up of a thalassaemia centre, an educational campaign for population screening for thalassaemia trait, for the need of prenatal diagnosis of affected mothers, in addition to better clinical management for existing TM patients. An outline of the organization health structure, the collaborating bodies involved in the prevention, diagnosis and treatment of thalassaemia in Cyprus as well as a historic perspective is shown in Figure 1.

The plan for the prevention of births of TM babies includes the option of prenatal diagnosis, which provides an opportunity to the pregnant mother to decide about the course of the pregnancy, usually before the 3rd month of pregnancy. In the case of birth of a TM child, the survival prospects are directly related to the treatment options available at the country of residence. Non transfused TM patients die from ineffective erythropoiesis of normal red blood cells and related complications usually by the age of 2-7 years. If regular red blood cell transfusions are available from matched blood donors, survival is expected to increase to about 15-20 years. At this stage TM patients usually die from excess iron accumulation and iron overload toxicity induced organ damage. Cardiac failure due to excess iron deposition in the heart is the major cause of death in regularly transfused TM patients

who do not receive adequate iron chelation therapy[20-23].

Life expectancy in TM increases following the application of effective chelation therapy within a period of a few years having started transfusions. A few compliant TM patients who adhered to the recommended protocol of sc deferoxamine (DF) (40-60 mg/kg per day, at least 5 d per week) were treated successfully since birth and have now exceeded 50 years of age. Compliance with sc DF for the majority of TM patients is poor and the average life expectancy is much shorter. Within this context, before deferiprone (L1) was introduced the mean life span of TM patients in the United Kingdom, was estimated to be 30-35 years[24]. In contrast, following the introduction of L1 in 1999, an improvement in compliance and efficacy as well as a reduction of cardiac deaths was observed in Cyprus and many other countries[25-29]. The prospects of compliance in relation to chelation therapy have also increased following the introduction of deferasirox (DFRA) in 2007. Chelation therapy with DFRA may mostly benefit TM patients who are intolerant or have other complications with both DF and L1 chelation therapy[18]. Several aspects are influencing the morbidity and mortality of TM patients worldwide including the efficacy, toxicity, availability and cost of all chelating drugs. In particular, access to these drugs may especially influence the overall survival of TM patients most of who live in the developing countries[18].

EPIDEMIOLOGY, TREATMENT AND SURVIVAL OF FRIEDREICH ATAXIA PATIENTS

FA is the most common autosomal recessive ataxia condition. It was identified by the German physician Nikolaus Friedreich in 1863 and only recently in 1996 the genetic mutation as well as the affected protein namely frataxin was identified and characterized[30-32]. This neurodegenerative inherited disease related to low production of frataxin appears to cause abnormalities in iron metabolism including the accumulation of iron in the mitochondria and consequential pathological complications of different severity in various organs. In particular, it causes progressive damage to both the central and peripheral nervous system resulting in a variety of symptoms ranging from gait disturbance and speech problems to diabetes and heart disease[3,4,33,34].

The geographic distribution and prevalence of FA is mainly in countries with Caucasian populations. It accounts for almost 50% of all the cases of hereditary ataxia. The FA carriers in Europe are estimated to be about 1:90, where in the United States it affects 1 in 50000 people. Apart from Europe and the United States, cases of FA have been recorded in the indigenous population of Mexico, Iran and India[35-37]. However, FA appears not to exist among the indigenous populations of sub-Saharan Africans, American Indians, Chinese, Japanese and Southeast Asians[38].

The FA carrier rate in Cyprus is estimated to be about 1:10, where in a cluster in the villages Kathikas and Arodhes in the Paphos district of Cyprus it is 1:5 or 1:6[10,39]. A total of 26 patients with FA were identified in Cyprus in a study in 2009[11].

There are individual variations in the onset and the severity of FA, which is characterised by progressive tissue and organ damage and significant morbidity. The onset of FA is early and almost always present before the age of 20 years. Loss of ambulation occurs about 15 years after the onset of the disease. More than 95% of patients are wheelchair bound by the age of 45 years. Frequent clinical findings of FA include the incoordination of limb movements, impairment of position and vibration, dysarthria, nystagmus, diminished or absent tendon reflexes, scoliosis, *etc*. In general, FA has progressive multipathological effects with a life span of approximately 30-40 years and the most frequently reported causes of death are diabetes mellitus and cardiomyopathy[3,4,33,34].

The socioeconomic consequences for the FA patients and their families and the costs associated with the treatment and care of these patients requires state intervention including steps for its prevention, diagnosis and treatment at the local level and worldwide. Within this context, preliminary scientific and epidemiological evidence prompted the introduction of government measures for the prevention or decrease in the number of births of FA patients in Cyprus similar to the adopted model of TM. These prevention measures were concentrated in the Paphos district where most of the patients and carriers of FA were previously identified and also to descendants from the Paphos district living elsewhere in Cyprus[11].

THE ROLE OF DIAGNOSIS AND PREVENTION OF THALASSAEMIA AND FRIEDREICH ATAXIA IN CYPRUS

Prenatal and antenatal diagnoses are widely used for the prevention of many inherited diseases with serious or fatal clinical complications, which are endemic in certain areas or ethnic groups. Both TM and FA belong to this category of diseases and Cyprus has the highest rate of asymptomatic heterozygote carriers of these diseases in comparison to any other nation. Within this context the prevention programme for TM started about 35 years ago whereas for FA it was recently adopted by the government in 2011 using the same guidelines as for TM.

In Cyprus the fate of TM patients prior to independence in 1960 was similar to that of a large majority of TM patients in the developing countries today, namely early death at 2-7 years due to lack of blood transfusions and other treatments. Following the independence of Cyprus and the initiation of a transfusion programme the survival rate of the TM patients increased. However, blood supplies were insufficient due to the high demand and the high annual birth rate of TM children at that period which was about 70 new cases per year[7].

The alarming rate of TM births and the socioeconomic consequences for the small population of Cyprus at that period prompted the initiation of new measures by the government in conjunction with institutions and organisations that could facilitate the process of prevention and the improvement of the treatment of patients.

Several initiatives including educational and publicity measures were taken to inform the general public of the campaign for the prevention of births of TM children and its implications in the families affected and society in general. Within this context a programme for the prevention of TM has been operating in Cyprus since 1973[7]. However, the major and most effective strategy for prevention was founded on thalassaemia trait compulsory screening for premarital couples. The strategy of compulsory screening was introduced in 1983, after an agreement between the government of Cyprus and the church, which carried out almost all weddings at that time. Accordingly a medical certificate was required by the church from all the premarital couples for confirming their thalassaemia status before proceeding to marriage.

In the meantime, the doctor managing the thalassaemia clinic where the diagnosis was carried out had to screen and inform the premarital couples of the possibility of having children with TM if they were both thalassaemia carriers and also to inform them of the available options to them including the option to decide the course of the pregnancy. Following marriage the prevention programme included genetic counselling and screening tests which were offered only for couples with thalassaemia trait, including chorionic villus sampling at about 11 wk of gestation of the pregnant mother[40,41]. If the test indicated a TM foetus, then the various options available were discussed and the final choice made.

Recent TM preventive measures for couples with thalassaemia trait included other possibilities, for example the pre-implantation diagnosis procedure[40]. This procedure was brought in to effect in Cyprus in the last 17 years and involves *in vitro* fertilisation for the analysis of early embryos and also the selection of an egg free of haemoglobin disorders. Following selection, further procedures include fertilisation of the egg in the laboratory and implantation into the womb[40,42]. Another possible option for future application is *in utero* allogeneic bone marrow transplantation.

The screening for thalassaemia trait is in most cases relatively simple and inexpensive, involving the electrophoresis of haemoglobin from blood samples[43-46]. Similarly, a blood sample is used in the case of FA screening but a more expensive DNA analysis is required to identify an FA carrier[47-49]. The strategy adopted for screening for the FA trait status involves in the first instance only persons with origins from the areas in Paphos where high prevalence of FA was identified[11].

The screening for thalassaemia carriers and the introduction of prenatal and antenatal diagnosis resulted in a significant drop in the number of births of patients with TM. The birth rate of TM children in Cyprus between 1970 and 1980 was estimated to be about 30-50 per year and a total of about 400 TM births were recorded. Following the implementation of the government and church policy on thalassaemia in 1983, the birth rate of TM babies between 1984 and 1994 dropped to about 25, *i.e.*, a rate 2-3 per year[7-9].

However, new trends have arisen in the last 20 years in Cyprus including major demographic changes, which had a major influenced in the birth rate of TM patients. It is estimated that 20% of the people residing in Cyprus are foreign workers and the total population has increased to approximately 1 million. Cyprus became a member country of the EU in 2004 and as a result of implementation of EU laws a medical certificate for thalassaemia is not obligatory for civic weddings, unlike church weddings[9]. Similarly, many couples are deciding to have TM children instead of abortion, because of emotional, religious and ethical reasons and also as a result of the newly improved treatments, which lead to an increase in the life expectancy of TM patients. As a result of these trends and also the marriage of Cypriots with non-Cypriot carriers the number of births of TM children has increased slightly in the last few years[50]. It is estimated that there were about 25 births of TM children from 2005 to 2011, *i.e.*, a rate of 3-4 per year. The trend on the birth rate of TM patients is set to persist at present, unless the law on civic weddings is changed and one similar to that of church weddings is adopted[9]. In the meantime, further reduction in the birth rate of TM children in Cyprus is expected following the recent introduction of other techniques such as that of the pre-implantation diagnosis. This procedure is costly but allows the selection of a non TM foetus by parents with thalassaemia trait or by parents where one is a TM patient and the other has the thalassaemia trait[42].

The prevention programme for FA is similar to TM but with slight modifications. The major difference is that the screening programme for FA carriers is concentrated in premarital couples in the Paphos district and individuals living in other areas of Cyprus but originating from the Paphos district. In a previous study involving the Paphos district a total of 1050 persons aged 18 and over were screened and 98 (9.33%) FA carriers were identified[11]. More FA carriers have been identified since 2011, when the government prenatal screening programme for FA on premarital members of couples originating from Paphos was initiated. In this case the partners of those identified as FA carriers were also screened.

While major efforts have been taken in Cyprus for the diagnosis of carriers of TM and FA and the prevention of births of TM and FA patients, there are still a small number of births with these inherited diseases and especially of TM patients every year. The small number of births of TM patients is related to cases of non prenatal screening and the choice of parents to have a TM child. Within this context the treatment of the newly born and existing patients is another continuous challenge in the day to day running of the thalassaemia clinics and the

Table 1 Biochemical and clinical monitoring of Thalassaemia major patients

Determination of thalassaemia mutation and blood group (ABO, rhesus)
Vaccination: Flu, pneumonia, haemophilous influenza, meningitis a + c and hepatitis B
Cardiac: Physical examination, echocardiography, holter, stress test, muga scan, (once a year or earlier if required)
Liver and kidney function tests, urate, cholesterol, triglycerides, Ca, PO4, fasting glucose test and prothrombin time (every six months)
Blood glucose tolerance test and screening for hepatitis B antibodies, hepatitis C and HIV (every year)
Bone densitometry, chest X-ray, Endocrine (T3, T4, TSH, PTH), ophthalmology, ear, nose, throat and echo of the abdomen examination (every year)
Iron load estimation: Serum ferritin and Zn (every 3-4 mo), serum ferritin for patients with normal physiological iron range (every month), MRI T2 and T2* of the heart and liver (once a year)
Full blood count (every 1-1.5 wk for those treated with L1 and L1/DF combination)
Supporting specialist therapy team: Cardiologist, endocrinologist, haematologist, paediatrician, dietician, gynaecologist, psychologist. Other clinical faculties if required

The table was adapted from ref. [9]. HIV: Human immunodeficiency virus; MRI: Magnetic resonance imaging; DF: Deferoxamine; TSH: Thyroid-stimulating hormone; PTH: Parathyroid hormone.

government health structure adopted in general.

ORGANISED HEALTH STRUCTURES FOR THE TREATMENT OF THALASSAEMIA AND FRIEDREICH ATAXIA PATIENTS IN CYPRUS

The developmental model of an organised health structure for the prevention, diagnosis and treatment of TM in Cyprus was primarily based on an initiative of the parents of TM children and also the physicians in charge of the patients, other scientists, the church, the government and other non governmental organisations (Figure 1). The cornerstone of the health structure was based on the organisation of the Cypriot thalassaemia society and the blood donors association in 1977. Further milestones in the development of an organised health structure was the organisation in Nicosia (the capital of Cyprus) of the thalassaemia centre in 1980 and thereafter the gradual evolution of the thalassaemia clinics in each of the districts of Cyprus in 1993.

In the postnatal phase an organised health structure for the treatment of TM patients in Cyprus involving many health sectors and other organisations has been designed and developed over many years with the centre of activity based in the district thalassaemia clinics (Figure 1). The structure was based on the main form of treatment of TM patients, namely chronic red blood cell transfusions and iron chelation therapy. A continuous programme of biochemical and clinical monitoring involving many common laboratory tests and other clinical specialists was organised by the thalassaemia clinics (Table 1).

A major aspect of the treatment and the survival of TM patients is blood transfusion. Blood donation and collection in Cyprus is organized successfully by the volunteer blood donor co-ordination committee in conjunction with the blood bank of the ministry of health. The blood donors co-ordination committee in Cyprus was initiated in 1977 by the local thalassaemia society and a number of clinicians with the slogan "give blood and save lives" with a reference to the TM patients[7-9].

Blood donation and collection was coordinated and progressively covered all towns and villages in all districts and community sectors in Cyprus. It involved both state and private organisations such as the army, schools, universities, police, banks, businesses and many others. Blood donation is strictly on a volunteer basis and is organised and carried out in each district by the local blood donor coordination committee involving thousands of blood donors. It is a purely volunteer organization with no financial budget or treasurer. Blood supplies are generally sufficient but in the summer holidays for example problems may arise in blood donation and blood supply may be at a shortage. In the latter case the state media call for an emergency blood donation of the relevant blood groups in shortage to be donated at the nearest district hospital.

Continuous biochemical and clinical monitoring as well as supportive specialist care involving a multi-disciplinary team is available in all the thalassaemia clinics in Cyprus for the follow up treatment of TM patients (Table 1). The multi-disciplinary clinical team include almost all clinical sectors such as specialists in cardiology, endocrinology, haematology, paediatrics, nutrition, gynaecology, psychology, *etc*.[9].

Iron chelation therapy introduced within a few years of beginning the red blood cell transfusions is essential for the survival of TM patients. In the absence of iron chelation therapy the life expectancy of TM patients is less than 20 years. Under these circumstances TM patients usually die from excess iron accumulation and organ damage. In particular cardiac failure due to excess iron deposition in the heart is the major cause of death in regularly transfused TM patients who do not receive effective iron chelation therapy[20-24].

Iron chelation therapy is widely available for both the use of DF and L1 and also in many cases for DFRA in different countries including Cyprus[51]. The T2 and T2* relaxation time magnetic resonance imaging techniques have been used over the past decade for the routine diagnosis of excess iron load in the liver, the heart and also other organs[52-56]. Monitoring of serum ferritin levels is also routinely used for estimating body iron overload.

Organ function and other biochemical tests are also routinely performed at different intervals as shown in Table 1.

Bone marrow transplantation has also been considered as a therapeutic option especially for young patients (usually under 7 years) when a matched human leukocyte antigen sibling is identified by the bone marrow transplantation laboratory. It is estimated that a total of about 20 Cypriot TM patients received bone marrow transplantations and in most cases were successful except for one death and two graft rejections[9]. Similar results were reported in TM clinics in other countries[57-60].

Treatment problems and other complications associated with the life of TM patients are frequently assessed by arranging interviews between the clinician in charge of the thalassaemia clinic and each TM patient around every other week[9].

The small number of FA patients by comparison to the large number of TM patients in Cyprus required the adoption of a smaller different organisational health structure for treatment provision in FA. Despite the abscence of effective therapy in FA, major efforts are directed towards the therapy of the symptoms of FA patients. Clinical and biochemical monitoring, as well as supportive therapy for the FA patients is carried out in a specialised neurology centre in Nicosia the capital of Cyprus[11,39,61].

In relation to treatment it appears that gross body or focal iron overload toxicity is the main cause of death in both TM and FA. A better understanding of the normal iron metabolic pathways and the detection and characterisation of the iron abnormalities and iron overload toxicity in both TM and FA can lead to effective treatments and reduction of the morbidity and mortality in these diseases.

IRON METABOLISM IN NORMAL AND ABNORMAL CONDITIONS INCLUDING THALASSAEMIA AND FRIEDREICH ATAXIA

Iron is an essential metal required by all mammalian cells for many biological processes and normal physiological functions. The solubility of ferric (Fe^{3+}) iron in aqueous solution at pH 7.4 is negligible (10^{-18} mol/L) and iron precipitation rapidly occurs in biological media in the absence of chelating proteins or low molecular weight (LMwt) chelators. Ferric iron is deposited in cells in a polynuclear form as ferritin, which is water soluble and haemosiderin which is not water soluble. Ferrous (Fe^{2+}) iron is more soluble than ferric iron in physiological conditions. In biological systems ferrous iron is found in mononuclear form bound to proteins but not in polynuclear form as precipitates or deposits. Ferrous iron is a major catalyst of free radical reactions. In contrast, ferritin and other protein bound iron does not appear to facilitate the catalysis of free radical reactions. However, haemosiderin and haemosiderin-like ferric iron deposits (focal iron), as well as other forms of labile, LMwt ferric iron are considered to be toxic. The latter provide a continuous source of catalytic iron which under certain conditions can readily give rise to free radical reactions and cascades leading to oxidative stress damage.

There are many conditions associated with iron metabolic imbalance, which are caused by genetic, nutritional, iatrogenic, environmental, disease and other factors and their combinations[5,13]. These factors can cause deficiency or overload or abnormal distribution of iron and a corresponding pathological effect from the molecular to tissue levels[5,13,62]. Examples of such changes in iron metabolic disorders is nutritional iron deficiency anaemia, idiopathic haemochromatosis which is a genetic iron overloading condition, Hallervorden-Spatz syndrome where iron accumulation in the brain is observed, anaemia of chronic disease where iron is diverted and deposited in macrophages of the reticuloendothelial system, sideroblastic anaemia where iron is deposited in ring sideroblasts, FA where excess iron is deposited in mitochondria, atransferrinemia where the iron transport protein transferrin is not produced leading to abnormal iron distribution and many others[5,13]. Abnormalities in iron metabolism can also be observed in relation to changes in the many proteins involved in the regulation of iron metabolism and of changes involving iron containing proteins including the haemoglobinopathies[1,5,13].

Body iron levels and iron distribution in the organs are governed under normal conditions by homeostatic controls of iron uptake, distribution and storage. In general, these levels are regulated by dietary iron absorption in the gastrointestinal tract, the erythropoietic activity of the bone marrow and the rate of iron excretion[5,13,62]. The absorption of iron mostly takes place from the intestine and is regulated by several metabolic pathways involving regulatory proteins such as hepcidin and ferroportin[63]. Following the uptake of iron into the enterocytes from the gut lumen, iron is then taken by plasma transferrin and transported in blood for delivery to all the cells of all tissues[63,64].

The total body iron of normal adult humans is estimated at 3-5 g and is present in different forms mainly as haemoglobin (58%), which is found in red blood cells, myoglobin (9%) which is found in muscle tissue and also as intracellular ferritin/haemosiderin (30%) which is found mainly in the liver but also other organs (15-17). The uptake, transport and distribution of iron is tightly controlled. For example, the uptake of iron from transferrin by cells and its storage intracellularly is regulated by specific iron regulatory proteins based on the translational control of the transferrin receptor synthesis at the cell surface and also that of intracellular ferritin, as well as by hepcidin[5,13].

The amount of iron delivered to cells is mainly determined by the number of transferrin receptors and also the iron saturation of transferrin. Transferrin iron saturation under normal physiological conditions is 25%-35%. However, in transfusional iron overload or primary hae-

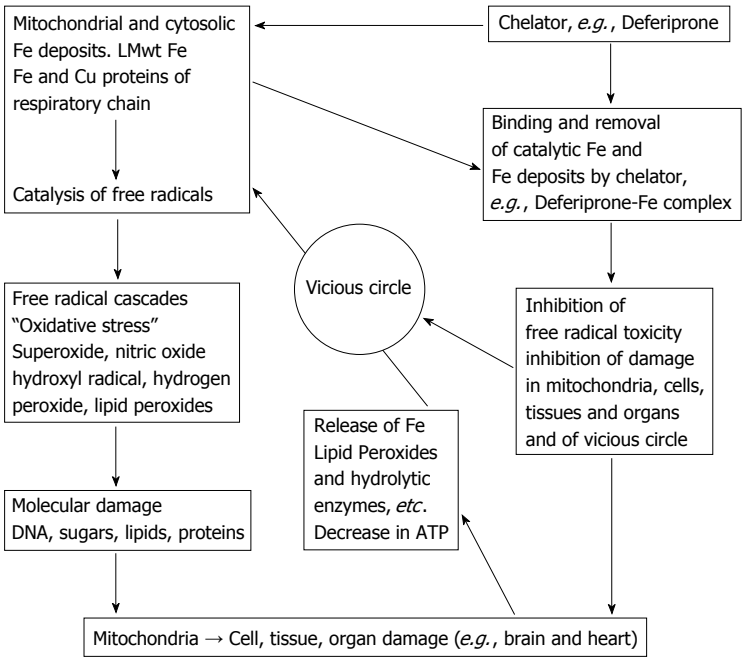

Figure 2 The mode of chelating and antioxidant activity of deferiprone in Friedreich Ataxia. Deferiprone can chelate intracellular and intramitochondrial iron deposits and labile low molecular weight (LMwt) iron, which are responsible for the catalytic formation of toxic free radicals and toxic byproducts. It can inhibit iron toxicity related damage to the heart and brain of Friedreich ataxia patients.

mochromatosis the saturation of transferrin with iron is much higher and usually exceeds 100%. At this stage where transferrin is saturated with iron, non-transferrin bound iron can be detected in the plasma[65-67].

The iron released from the continuous breakdown of effete red blood cells and the catabolism of haemoglobin, as well as of other cells of the body is transported in the blood by transferrin, which in conjunction with hepcidin and other regulatory proteins of iron metabolism maintains equilibrium between the sites of iron absorption from the gut, intracellular storage and also utilisation. Iron uptake by cells is accomplished by the binding of transferrin iron to a transferrin receptor on the cell surface. Usually two molecules of monoferric or diferric transferrin is bound to each transferrin receptor, which subsequently is incorporated into the cell within an endosome. The release of iron from transferrin is carried out through acidification of the endosome from pH 7.4 to 5.6. The iron molecules released are in a LMwt form and are incorporated in the "transit iron pool". Iron is then transported to all parts of the cell including the mitochondria, for storage in ferritin or haemosiderin and for incorporation into apoproteins for the formation of iron containing proteins.

The LMwt intracellular transit iron pool is thought to be composed mainly by LMwt chelator iron complexes[62,68]. Some LMwt naturally occurring chelators are found in cells, *e.g.*, citrate, ATP, ADP, glutathione, amino acids and some absorbed from food eg phytic acid, polyphenols and ascorbate. These and other similar molecules are thought to be involved in the transfer of iron in different intracellular compartments affecting intracellular iron metabolism. For example, they can form ternary metal complexes with apo-proteins or facilitate the incorporation of iron into the metal domain of proteins. The mechanisms and processes of iron uptake, release and exchange are governed by the same kinetic and thermodynamic parameters as for those applied to other chelators and their iron or other metal complexes[68]. Within this context, it is anticipated that chelators present at high concentrations eg citrate (10 mmol/L, in plasma) and glutathione in cells (5 mmol/L in liver cells) may play a significant role in these processes.

One of the most important functions of iron and some iron containing proteins is the catalysis of free radical reactions, and formation of related byproducts which are essential for physiological processes[69]. Free radicals and other nitrogen and oxygen activated products such as the nitrogen oxide, superoxide, hydroxyl radical, lipid peroxides and hydrogen peroxide are constantly and continuously generated in aerobic organisms during normal metabolism and physiological functions and also in response to both internal and external stimuli. However, if free radical reactions and related by-products are continuously overproduced and are not regulated or controlled by the antioxidant pathways and molecules, this can cause free radical toxicity and damage from the molecular level to the organ level as shown in many diseases including TM and FA[69-71].

The catalytic effects of iron can cause free radical toxicity cascades and biomolecular damage including damage to sugars, lipids, proteins, DNA and also widespread subcellular, cellular, tissue and organ damage (Figure 2). Such free radical cascades are not sufficiently neutralized by antioxidants but can usually be prevented by iron binding or chelation[69,70].

Iron absorption and transferrin iron delivery appears to be normal in FA patients, with the general iron metabolic parameters such as serum ferritin and transferrin iron saturation to be within the normal physiological ranges. The frataxin abnormality appears to affect selectively certain organs and tissues and the damage to be manifested slowly over many years, in a time scale similar to the case of iron overload in hereditary haemochromatosis patients[13,62,63]. In the affected cells the frataxin abnormality appears to cause the increased uptake and deposition of iron in mitochondria.

The transport of iron from the cytosol to the mitochondria and its incorporation into the apoproteins for the formation of mitochondrial iron containing proteins or incorporation into mitochondrial ferritin is not well characterised or understood in FA[72-74]. Similarly, there are controversies as to the presence of only mitochondrial iron deposits or both cytosolic and mitochondrial iron deposits in the affected cells. Accordingly, some investigators have reported the presence of excess iron in mitochondria but not the cytosol and others excess iron deposits in both sites[75,76].

A possible pathway can be suggested to explain these differences. Within this context and under normal conditions a pathway is operating involving the transport of LMwt iron complexes from the cytosol or protein bound iron, across the mitochondrial membrane and the formation of "intra-mitochondrial transit LMwt iron pool" before iron incorporation into the mitochondrial apoproteins or mitochondrial ferritin.

Incorporation of iron into ferritin including mitochondrial ferritin is usually in the reduced mononuclear ferrous form which is then oxidised and polymerised inside ferritin in an oxohydroxide iron phosphate polynuclear form[77,78]. Polynuclear iron cannot be incorporated inside ferritin but can be deposited as a haemosiderin-like polynuclear iron in the cytosol or mitochondria. In the latter case deposits of iron oxyhydroxy phosphate and sulphur polymers were identified in an animal model of FA, which were different from the forms of iron incorporated into mitochondrial ferritin[73]. Similarly, haemosiderin-like iron can also be formed from the breakdown of the ferritin protein shell and exposure of the intra-ferritin iron core[77,78]. Based on these observarions two different stages of iron deposition appear to be taking place in FA in a time dependent manner. In the initial stages there is an increased uptake of cytosolic iron into mitochondria, where cytosolic iron deficiency may also occur[75]. In the second stage, saturation of mitochondria with iron can cause progressive increase in cytosolic iron accumulation and the formation of iron deposits in the cytosol[76].

The iron deposition process in FA may be facilitated by several factors including the low production of ATP in mitochondria which decreases both the intra-mitochondrial LMwt transit iron pool and iron incorporation in mitochondrial ferritin, thus leading progressively to the increased polymerisation and deposition of haemosiderin-like iron in mitochondria[79]. Similarly, decreased production of ATP could cause a decrease in the cytosolic LMwt leading to progressive iron polymerisation and deposition in cytosol[78].

An additional factor that may facilitate excess iron deposition in mitochondria is the presence of oxidative environment which causes iron oxidation and precipitation. Ferrous iron is more water soluble than ferric iron and the former can be oxidised and precipitate forming a haemosiderin-like polynuclear iron inside the mitochondria. Other similar factors may possibly be involved in the intra-mitochondrial recycling of iron, where for example iron re-utilisation from the turnover of mitochondrial iron containing proteins is blocked and it undergoes polymerisation and accumulation as iron deposits in the mitochondria[80].

Another possibility for the excess iron deposition in mitochondria is the presence of a mechanism of iron loading of mitochondria similar to that observed in primary or hereditary haemochromatosis involving the malfunction of regulatory proteins like herceptin and ferroportin. In the case of chronic iron overload disorders, iron deposition progressively results in increased formation of haemosiderin polynuclear iron complexes which are difficult to mobilise. In such cases polynuclear iron removal from ferritin, haemosiderin and other forms of iron deposits follows the "last in-first out" general principle. Similarly, newly formed polynuclear iron formations and precipitates can be mobilised faster and more efficiently by chelation than older ones[81].

In addition to the toxic iron deposits and the increased oxidative stress observed in the mitochondria of FA patients, low frataxin levels can also cause other metabolic abnormalities such as the insufficient production of iron-sulfur clusters that are required for mitochondrial electron transport and energy transduction, as well as the functioning of aconitase and other iron containing enzymes or iron related metabolic pathways[79]. The distortion of these processes and in particular of mitochondrial electron transport is an additional powerful source of free radical production, which if not controlled can cause free radical cascades, toxicity and damage (Figure 2).

Organs such as the heart and the brain with high energy consumption and increased numbers of mitochondria appear to be particularly sensitive to these forms of abnormalities. Cardiac, brain and other organ damage, resulting in insufficient organ functioning levels is the overall cause of increased morbidity and mortality in FA.

PATHOGENIC EFFECTS OF THE IRON METABOLIC ABNORMALITIES IN THALASSAEMIA AND FRIEDREICH ATAXIA

There are many differences between TM and FA in relation to the iron metabolic pathways involved, such as the cellular and body distribution of iron, the origin and quantity of iron accumulated, the biochemical parameters

related to iron imbalance, the target organs of iron toxicity and many others. In assessing these differences it is important to specify some of the pathways, properties and characteristics of iron metabolism which are relevant to the diagnosis and treatment of each of these two conditions. Within this context the characterisation of the molecular pathways involved under normal physiological conditions and the pathological effects observed in both TM and FA, including the pathogenesis of gross or focal iron overload toxicity are considered essential in the understanding of the mechanisms involved in both diseases and also for the proposed treatment options.

Gross body iron overload is one of the most common metal overload toxicity condition which can be caused by increased iron absorption from the gut (primary haemochromatosis) or regular transfusions of red blood cells (secondary haemochromatosis) or a combination of both these two processes. Thalassaemia patients or patients with other refractory anaemias are regularly transfused every 1-4 wk, usually with 1-3 units (1 unit = 200 mg of iron) of red blood cells for maintaining haemoglobin levels above 9-10 mg/dL. Iron overload in refractory anaemias including TM has the highest morbidity and mortality rate worldwide in comparison to other iron or other metal overloading condition. One of the most seriously affected group of iron overloaded transfused patients are the TM patients.

The accumulated iron from transfusions cannot be excreted and is stored intracellularly as ferritin and especially as haemosiderin. The latter protein increases in concentration in some main organs, particularly in the liver as well as the heart and spleen of the transfused TM patients. Previous electron microscopy studies in cardiomyocytes and hepatocytes of TM patients have shown that in iron overloaded conditions iron loaded ferritin arrays are formed intracellularly mainly in primary lysosomes and haemosiderin iron aggregates in secondary lysosomes[21,82,83]. Further ultrastructural observations suggest that in heavy iron overload in TM there are some iron-laden lysosomes which are ruptured into the cell sap and may be a cause of toxicity. Other forms of subcellular damage which are identified for example in cardiomyocytes of TM who suffered congestive cardiac failure, include the presence of large cytoplasmic vacuoles, increase in the electron density of nuclei and increased amounts of heterochromatin, substantial loss of myofilaments, swollen mitochondria with loss of their cristae but with no iron deposits within them[84].

The damaging effects of iron overload and the rupture of iron-laden lysosomes into the cell sap can also cause the release of hydrolytic enzymes and potentially toxic forms of labile iron. The latter can catalyse the production of free radical cascades and cause further free radical damage which can progressively lead to a vicious cycle of further cellular, tissue and organ damage (Figure 2)[69,70].

Iron overload and toxicity causes organ damage, which is generally detectable when approximately 50-100 units of red blood cells have been transfused. This damage is reversible in the early stages but in many cases is so extensive that it can become irreversible[84]. Regularly transfused iron loaded TM patients usually die from iron overload related cardiomyopathy[20,23,26]. The possibility of congestive cardiac failure due to iron overload deposition in the heart is observed in TM patients with cardiac magnetic resonance imaging (MRI) T2* values of lower than 9 ms[53-55]. In addition to heart complications, other forms of iron overload toxicity include liver fibrosis and cirrhosis, splenomegaly, endocrine organ damage leading to diabetes, retarded growth and sexual immaturation. A major difference between TM and FA is that there is no iron accumulation in the brain or related toxic side effects involving the nervous system in TM patients.

Different molecular factors and pathological effects due to iron toxicity are observed in FA patients. Friedreich ataxia is an autosomal recessive disease like TM, which is caused by a mutation involving homozygous GAA trinucleotide intronic repeat expansions in the gene in chromosome 9, which encodes the mitochondrial matrix protein frataxin[3,4]. The repeat expansions interfere with transcription of the protein frataxin. About 97% of patients with FA have expansions of a GAA repeat in the first intron of both alleles of the frataxin gene. In normal individuals the alleles of the frataxin gene have 36 or fewer GAA repeats, while in the FA disease the alleles have from approximately 70 to about 1700 GAA repeats. It appears that regarding pathogenesis the age of onset correlates inversely to the size of the GAA repeat of the expansions, and directly with the rate of disease progression.

Frataxin appears to play an important role in mitochondrial iron homeostasis and is involved in the biosynthesis and structural assembly of iron-sulfur cluster proteins and also in haem synthesis. It is severely reduced to about 5%-20% of normal in FA patients but the level of its deficiency needed to cause disease is still unknown. The decreased production of frataxin in FA patients appears to be associated with many clinical abnormalities[3,4].

Variations in the levels of frataxin are observed between different tissues and the tissues mostly affected are expected to have severely reduced levels. A detailed description of the symptoms or signs and the anatomic localization involved is reviewed elsewhere[85]. The severity of the disease in FA and other inherited diseases seems to also depend on many other factors. Variations in compensatory mechanisms and alternative metabolic pathways which are in operation in affected cells and tissues may contribute to minimising the severity of FA[85]. Similar variations are observed in other inherited and metabolic diseases including TM and hereditary haemochromatosis[13,62,85-87].

Deficit of frataxin is mostly related to many abnormalities associated with mitochondrial structure and function. The main and characteristic abnormality in FA is an increased iron accumulation and deposition in the mitochondria matrix and a corresponding increase in

oxidative stress[3,4,88,89]. There is also deficit of mitochondrial respiratory chain complex activities and impairment of tissue energy metabolism, decrease in production of ATP, effects on the function of aconitase and other abnormalities which lead to mitochondrial misfunction, including also to a further increase in the production of free radicals and other reactive oxygen species. Increased cytosolic iron in cells of specific tissues is observed despite that the general body iron stores and metabolic parameters are within the normal physiological range in FA[90].

Histopathological and MRI studies have shown that iron primarily accumulates in heart muscle, dentate nuclei (spinocerebellar tracts) and spinal cord of patients with FA[76,91,92]. In these studies, focal localised iron deposits have been detected by MRI T2* in the brain and the heart of FA patients[76,91]. In histopathological studies stainable positive iron deposits have been identified in the cardiomyocytes in autopsy and biopsy specimens of FA patients[76]. It is important to note that patients with short GAA repeat expansions and long survival have neither heart disease nor focal localised iron deposits.

Many contributory factors appear to be involved in the observed variation in the onset and progression of disease in FA patients. In addition to the size of the GAA repeat expansions and the focal localised iron deposits in the heart and brain, other such factors include the possibility of frataxin isoforms, distribution of iron in other tissues or organs, dietary habits and many other[62,63,93]. The pathogenesis of the disease and the high level of toxicity observed mainly in the heart and the brain of FA patients may also be related to the high requirements and consumption but insufficient utilization of oxygen due to mitochondrial malfunctioning in these main target organs. Other organs appear not to be affected to the same extent because of reduced requirements, consumption and utilisation of oxygen.

There are many differences in the distribution and pathogenic effects of iron in TM and FA. In both conditions gross or focal iron overload deposits appear to be a major factor in the cause of the toxicity. Furthermore, in TM iron overload is in the form of ferritin and more so in the form of haemosiderin and situated in lysosomes in the cytosol and not the mitochondria[84]. In contrast, polynuclear iron deposits of mainly ferritin and haemosiderin have been identified in both the mitochondria and cytosol of cardiac and other biopsy samples of FA patients[76,88,89].

Despite that cardiomyopathy is the main cause of death in both diseases, iron accumulation in the hearts of patients with FA is overall much lower in comparison to TM patients. However, cardiomyocytes in FA appear to be more susceptible to toxicity and damage arising mainly from focal iron deposition, mitochondrial iron overload and respiratory chain protein malfunction in comparison to mainly cytosolic gross body iron overload deposition and toxicity in TM.

Iron deposition in the pancreas may also be partly the cause of the increased incidence of diabetes in both the TM and FA categories of affected patients[34,94,95]. Pancreatic damage due to iron overload is well documented in TM and in some cases it may be reversible with chelation therapy at the early stages, whereas in FA it is still under investigation[96].

Iron deposition in the cerebellum in the brain of FA patients, as well as in other parts of the central and peripheral nervous system may play a major part in the cause of the observed pathology of FA and especially the ataxia[85]. Neurons appear to be more susceptible to iron toxicity than other cells. In contrast iron accumulation in the hypophysis (pituitary gland) in the brain of TM patients affects body growth and delays puberty. No other parts of the brain or of the nervous system in general appear to accumulate and deposit iron in TM and no neurological effects have been reported in severely iron loaded TM patients.

In general, the clinical problems arising from the abnormalities of frataxin are not equally affecting the FA patients or the various organs with the exception of the heart, the brain and other parts of the nervous system[97]. For example the serum iron and haemoglobin levels of FA patients appear to be within the normal physiological range despite that haem synthesis is carried out in mitochondria mainly in the bone marrow and spleen[90]. This suggests that the haemopoietic tissues are not affected or targeted by the toxicity mechanisms related to low levels frataxin and also of other related abnormalities including iron deposition observed in the mitochondria and cytosol of FA patients[97].

Overall, the iron deposition rate in the mitochondria of the tissues affected in FA patients appears to be very slow. Similarly, the process of iron toxicity may take years to reach the stage for pathological symptoms to be expressed and identified. Similar time scales are observed in the deposition of excess gross body iron load, as well as the expression of iron toxicity and pathological symptoms in hereditary haemochromatosis patients[13].

Whatever the rate or mechanisms of iron accumulation and deposition in TM and FA, it appears that gross body or focal localised iron overload is the major source of pathogenesis and the major factor of the increased morbidity and mortality observed in both diseases. Within this context iron chelation is considered at present as the first line therapy for the treatment of TM and FA patients.

THE ROLE OF DEFERIPRONE IN CHANGING THALASSAEMIA FROM FATAL TO A CHRONIC DISEASE

Regularly transfused TM patients not receiving iron chelation therapy have a poor prognosis and usually die from congestive cardiac failure and other iron overload complications by the age of 20 years[20-24].

There are three main iron chelating drugs which are regularly used for the treatment of transfusional iron overload in TM and other conditions namely DF, L1

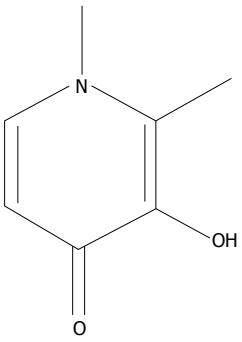

Figure 3 The chemical structure of the iron chelating drug deferiprone (L1). Deferiprone can bind iron through the two molecules of oxygen. It is a bidentate chelator and at physiological pH three molecules of L1 are used for binding one molecule of iron.

and DFRA. Both DF and L1 are generic drugs, whereas DFRA is a relatively new patented drug and all three are marketed in many countries worldwide[13].

Deferiprone was invented in 1981 in the United Kingdom and selected as a leading chelating compound out of about 150 related analogues and other chelating compounds (Figure 3)[13,98-101]. Many preclinical studies have shown promising results in the ability of L1 to mobilise iron from iron containing proteins, cells and animals and to prevent iron induced free radical toxicity[13,99-101].

The first clinical trials in iron loaded myelodysplasia and TM patients in 1987 in London, United Kingdom showed that oral L1 can cause equivalent iron excretion to subcutaneous DF[102,103]. The academic initiatives in the development of L1 continued and multicenter clinical trials were initiated by academic departments in many European countries, in Canada and in India, confirming the earlier results in London, United Kingdom[104-108]. The multicenter clinical trials involved mainly thalassaemia patients who were unable to receive DF due to toxicity, low compliance or both. Deferiprone was first registered in India in 1994 and then in the EU, Asian and other countries in 1999 and the United States in 2011[109].

The introduction of iron chelation therapy has increased the survival of TM patients. In a United Kingdom report prior to the introduction of L1 and DFRA the mean life expectancy of TM patients treated with DF was reported to be 35 years[24]. Deferoxamine was freely available to all TM patients in the United Kingdom and the main reasons of fatalities were iron overload cardiac failure due to non-compliance with the daily subcutaneous administration of DF and the inability of DF to remove effectively iron from the heart[24]. Similar outcomes with the DF treatment were observed in other countries such as Italy, Greece and Cyprus where DF was also freely available from the state to all TM patients[24-26].

Substantial reduction in morbidity and mortality has been observed since the introduction of L1 due to increased compliance and the effectiveness of L1 to remove excess cardiac iron. This improvement has been observed in many countries such as Cyprus, Greece, Italy, United Kingdom and Hong Kong where monotherapy of L1 or combinations with DF were used[25-29]. This was also encouraging evidence that iron overload toxicity is reversible and can be prevented in most cases especially when appropriate chelation therapy protocols using L1 and DF are introduced at an early age. Recent evidence suggests that the use of the appropriate chelation protocols and especially of specific combinations of L1 and DF could achieve the main aim of iron chelation therapy, namely the complete elimination of excess iron load and the associated iron overload toxicity complications[110].

However, the progress in the treatment of TM patients in developed countries including iron chelation therapy is not reflected in developing countries. It is estimated that more than 90% of TM patients worldwide, who mostly live in developing countries do not receive regular red blood transfusions and iron chelation therapy due to the high cost of treatment. Similar problems were encountered in Cyprus prior to 1977, when regular transfusions or chelation therapy were not accessible to the majority of patients due to the high cost, lack of facilities and lack of an organised health structure system.

Much progress has taken place in the treatment of TM and particularly regarding iron chelation therapy in the last 20 years in Cyprus that has changed the prognosis of TM from a fatal to a chronic disease. Since the introduction of L1 in Cyprus in 1999 many patients (70%) with poor compliance and adverse toxic effects to DF have either included L1 as a part of a combination protocol or switched over to L1 monotherapy[25]. The advances in chelation therapy with the introduction of L1 has led to the overall decrease in iron overload including serum ferritin levels, in improved compliance with chelation therapy, which was previously characterised as poor, as well as improved quality of life for patients[9,25].

The transition from a fatal to a chronic disease can be illustrated by the follow up of TM patients in the thalassaemia clinics of Cyprus. The TM patients undergo regular clinical and biochemical monitoring related to the complications of the underlying disease and the associated therapeutic applications including chelation therapy (Table 1)[9]. Similarly, the introduction of clinical studies and clinical trials involving TM patients in some of the clinics has played an important role in the development of general and personalised optimal therapies for the benefit of all patients[9,51].

Within this context clinical studies have shown that the most effective treatment of transfusional iron overload is the use of L1/DF combination protocols and particularly the International Committee On Chelation (ICOC) protocol (L1 at 75-100 mg/kg per day and DF at 40-60 mg/kg at least 3 d per week). This protocol appear to reduce serum ferritin and to increase MRI T2* of the heart and liver progressively to physiological normal levels[110-114]. Furthermore L1 monotherapy (85-100 mg/kg per day) appears to be sufficiently effective for maintaining the body iron stores at normal physiological levels in most TM patients[112,114]. This development is a major breakthrough in iron chelation therapy because it allevi-

ates TM patients from all the toxic side effects associated with iron overload toxicity and reduces substantially the overall morbidity and mortality to levels similar to those observed in the normal population[110,113].

An increase in compliance in TM patients was noted following the introduction of L1, in addition to the efficacy in iron removal. The majority of TM patients appear to receive satisfactory treatment with L1 in Cyprus either in combination with DF (> 50%) or as monotherapy (< 20%)[9,25]. Increase in compliance is also observed in TM patients receiving DFRA, despite that in some reports the efficacy in the overall iron removal and in particular iron removal from the heart is substantially reduced in comparison to the L1/DF combination[115,116]. Similarly, improved compliance and reduced episodes of local allergic reactions were also noted in many TM patients using an elastomeric pump as opposed to an electronic pump for the subcutaneous infusion of DF (< 20%)[9].

Important progress has also been observed following the introduction of the MRI T2* and T2 relaxation time techniques which have shown to be very valuable for monitoring excess iron load and for improving iron chelation therapy for the TM patients in Cyprus and other countries[9,52-56].

The most important finding in relation to chelation therapy in TM is that since the introduction of L1 the quality of life for most patients has greatly improved and most adult TM patients in Cyprus have developed professional careers, got married and had families. Characteristically about half of the 50 adult TM patients attending the thalassaemia clinic in Paphos have married, had in total 29 children and of note one female patient treated with L1 has successfully given birth to 4 children and also has two grandchildren[9].

The paradigm of Cyprus and the adopted organisational health structure for the prevention, diagnosis and treatment of TM including the protocols on iron chelation therapy with L1 in particular, could be used as an example to be followed by many countries where there is a high incidence of TM. Similarly, the high safety profile of L1 prompted its clinical application in many other non iron loaded conditions with focal localised iron deposits such as acute kidney disease, neurodegeneration with brain iron accumulation, Parkinson's disease and FA[117-120].

THE ROLE OF DEFERIPRONE IN THE TREATMENT OF FRIEDREICH ATAXIA

The general clinical care of FA patients is primarily concentrated on the treatment of the symptoms of the underlying disease. There is no established or effective treatment available for FA at present and no clear therapeutic targets or strategies developed to reverse the pathogenesis of the disease. Most efforts until recently were concentrated on the use of antioxidants for the treatment of the oxidative stress related pathology of FA. The most widely used antioxidants in FA are vitamin E, idebenone, coenzyme Q10, selenium and N-acetyl cysteine[121,122]. Despite that these antioxidants are used at random and in many cases for prophylaxis by FA patients the results are not encouraging and in most cases do not delay the onset or the progression of the disease[121,122].

The suggestion of the use of specific iron chelation therapy and especially L1 for the removal of excess iron from mitochondria for the prevention of pathogenesis and the treatment of FA in general was originally proposed in 2003[5]. The proposed mechanism for the mode of action of L1 was removal of localised excess deposited iron and inhibition of the iron induced catalytic activity and free radical cascades which caused increase in oxidative stress and cellular damage. This proposal was based on *in vitro* findings, animal studies and clinical results suggesting that L1 had access to all major organs and tissues including the heart and the brain[5,69,70]. The ability of L1 to remove excess deposited iron from the hearts of FA patients similar to TM patients, was also suggested since focal or gross iron overload appears to be the main cause of mortality in both diseases[5,69,70]. Similarly, it was also suggested that the application of L1 was a more appropriate therapy than the use of antioxidants for the toxicity arising from focal or localised excess deposited iron and also that the therapeutic options included drug combinations[5,69,70].

The suitability of L1 for clinical use in FA was until recently questioned, mainly because of lack of experience and knowledge on L1 chelation by those involved with the care of FA patients. Within this context a number of concerns were expressed regarding possible toxicities.

One of the major concerns in the use of iron chelating drugs in non iron loaded conditions including those with focal or localised excess deposited iron such as FA, Parkinson's disease, Alzheimer's disease and Hallevorden-Spatz syndrome is the induction of iron deficiency anaemia and other related toxicity. Despite that such hypotheses may be theoretically valid, each case should have been evaluated separately and background information on the clinical use of chelators thoroughly investigated. Within this context many wrong assumptions, expectations and conclusions can be made that may effect future investigations. In particular, the physicochemical, pharmacological and toxicological properties as well as the mode of action of chelators need to be taken into consideration for targeting each one of such conditions.

The specific properties of iron chelating drugs required for targeting iron toxicity and the risk/benefit assessment for use in each clinical condition have been previously reviewed[5,13]. Similarly, the suitability of L1 in FA and other non iron loaded conditions with focal or localised excess deposited iron as well as the concerns in relation to iron deficiency and related toxicity have also been previously addressed[5,69,70]. In such cases the interactions of chelators and especially L1 with tranferrin is essential in understanding the mechanisms and pathways involved[64,123].

In vitro and *in vivo* studies have previously shown that L1 can donate iron to unsaturated or partially saturated

tranferrin provided L1 is in the form of an iron complex and is present at a concentration of about the same level or lower than what is required to saturate tranferrin with iron[64,123]. Monitoring of plasma samples of normal individuals have shown that the oral administration of L1 causes a progressive increase of tranferrin iron saturation from the normal physiological range level (20%-35%) to a level of up to 80% over a period of 7 h[124]. This coincides with the results of pharmacokinetic studies where L1 has been shown to be cleared from plasma over a period of 6-7 h[67]. These findings suggest that L1 can mobilise iron from cytosolic intracellular storage sites or in the case of FA from focal iron deposits in mitochondria also and donate it to apotransferrin or monoferric transferrin in plasma, resulting in an increase of tranferrin iron saturation[64,124,125].

The above study suggests that the iron mobilised by L1 in individuals with normal body iron store levels including focal iron deposits in FA patients could be redistributed in the body *via* transferrin instead of being excreted. Measurement of urinary iron excretions have shown that usually only about 1-3 mg iron can be excreted by normal individuals of about 75 kg body weight, per a 3 g dose of L1[67,124-126]. In comparison, in iron loaded TM patients of approximately the same body weight, substantial increases in iron excretion of up to 71.5 mg can be observed by using the same dose of L1[67,123-126].

Despite that the net amount of iron excreted by L1 in normal individuals is small, continuous administration over prolonged periods of many months and years may cause iron deficiency, unless iron can be replaced from dietary sources or from iron supplements[64,123]. Monitoring of the biochemical parameters related to the iron status is particularly important for FA patients treated with L1 or other chelating drugs. The same monitoring applies for Parkinson's disease and other non iron loaded groups of patients, as well as for individuals using L1 as an antioxidant or anti-ageing drug, where administration of L1 may continue for many months and years[69,127].

Following the original proposal for the use of L1 in the treatment of FA several studies and clinical trials have been carried out confirming the selection and suitability of L1 as a promising therapeutic agent in this condition[5]. In cell studies using the HEK-293 FA model in which frataxin mRNA was knocked down, L1 (50 μmol/L) caused an increased in ATP production, mitochondrial membrane redox potential and resistance to staurosporine-induced apoptosis[128]. In the same model L1 outperformed DFRA, DF and salicylaldehyde isonicotinoyl hydrazone confirming its role as an effective iron chelator and antioxidant. The superiority of L1 in this model is based on its higher iron binding potential (log β3 = 36), the increased access to intracellular and intramitochondrial compartments, the iron mobilising and antioxidant properties and the efficient cellular exit of the L1 iron complex[13].

A number of clinical trials examining possible therapeutic effects of L1 in FA patients have been reported in the last few years. In one clinical trial nine Friedreich ataxia patients were treated with a dose of L1 of 20-30 mg/kg per day for 6 mo. In this study L1 has been shown to reduce excess toxic iron in the brain as determined by MRI T2*, and also to reduce ataxic gait and neuropathy in general[118]. Similar encouraging results were observed in patients with FA using a combination therapy of L1 with the antioxidant drug idebenone[129]. In another study 13 patients were treated with triple therapy using deferiprone at 5-25 mg/kg per day, idebenone at 10-20 mg/kg per day and riboflavin at 10-15 mg/kg per day for 15-45 mo[130]. Both neurological and heart function benefits were noted in some patients. Some adverse effects due to L1 were observed in four of the patients[130]. Furthermore, a double-blind, randomised, placebo-controlled phase 2 trial of L1 in a group of 80 individuals with FA was reported[6]. Full details of this study have not yet been published[6]. No significant overall changes in the ataxia scale scores in this study were observed, despite that improvement in posture, gait, and kinetic function were observed in some patients. Similarly, the L1 treatment was associated with cardiac improvement such as a decrease in the left ventricular mass. Decrease in serum ferritin levels was observed in a few patients. There was also one case of neutropenia which was resolved on L1 withdrawal[6].

Overall major therapeutic outcomes have been generally observed in FA patients treated with L1 during the clinical trials with both neurological and heart function improvements. It should be emphasised that complete clearance of excess cardiac iron in TM and brain iron in neurodegenerative disease patients has been previously achieved using higher doses of L1 over longer periods and the same result is expected in FA patients[112-114,119,131]. These optimistic results offer hope to FA patients who otherwise have no other effective treatment.

There are several concerns on the protocols used so far in FA patients including the low doses of L1 and the combinations with other antioxidant drugs. In particular, there is a scope of major improvements in the use of L1 in FA patients, including the prospects of personalised medicine. In the latter case the therapeutic targets have to be clearly defined and the appropriate L1 dose protocols selected based on the focal iron load in the brain, heart, pancreas and other organs, as well as the overall risk/benefit assessment in each patient. In general, the elimination of focal or localised iron deposits in the heart, the brain and other organs of FA patients are considered as a major therapeutic target in FA because it is a source of continuous toxicity and cause of pathological symptoms.

Previous clinical studies with L1 in non iron loaded patients or normal volunteers can serve as an example for designing appropriate treatment protocols of FA patients. For example, focal or labile iron deposits have also been targeted in kidney diseases patients. In clinical studies using doses of L1 of 50-75 mg/kg per day in about 50 non iron loaded patients with acute kidney disease for up to 9 mo, L1 improved kidney function, caused a de-

crease in proteinuria and no serious toxic side effects[117]. Overall L1 was well tolerated despite that the doses used were two or three times higher than those used in the FA studies. It is anticipated that similar higher doses of L1 at 50-75 mg/kg per day can also be used in most FA patients, especially those with increased focal iron deposits in the brain, heart and pancreas. The prospects of other improved therapies including chelator combination therapies, combinations with other drugs and the introduction of personalised medicine based on the focal iron deposits and the stage of the underlying disease should also be considered for future clinical investigations.

FUTURE PROSPECTS IN THE TREATMENT OF THALASSAEMIA AND FRIEDREICH ATAXIA

Major progress has been achieved in the diagnosis, prevention and treatment of TM and FA in the last two decades. The paradigm of Cyprus shows the way forward for the prevention and treatment of these pathological inherited diseases. However, these achievements have not yet been realised for most patients and in particular the vast majority of TM patients who live in developing countries. Similar developments have occurred in FA, but the lack of clear approaches and suitable strategies may undermine the efforts for prevention and effective therapeutic interventions, especially in relation to the removal of focal toxic iron deposits in the heart and the brain.

One of the major achievements of the last two decades is the substantial reduction in the births of TM children in Cyprus and many other countries worldwide using similar birth prevention measures. Public education policies and especially consultations with the heterozygote couples at risk of giving birth to TM children played a major role in this achievement. Similarly, the availability of prenatal and antenatal diagnoses substantially reduced the rate of births of TM children.

The quality of life and survival of TM patients have increased substantially in the last 20 years as a consequence of the introduction of effective therapeutic postnatal measures and especially diagnostic advances in the estimation of iron overload using the MRI T2* and T2 techniques and especially the effective treatment of iron overload with L1 and its combinations[86,132]. These changes and related improvements are more obvious in Cyprus and other countries where government health authority intervention is significant and appropriate by-laws as well as appropriate procedures are implemented using effective organisational health structures.

The organisational health structure adopted in Cyprus led to the decrease and almost the total elimination of births of TM children. The birth of a few TM children in Cyprus by those not adhering to the prenatal and antenatal screening programmes has highlighted the importance of the implementation of health policies related to the government's strategy for the prevention of thalassaemia. The introduction of other interventions such as preimplantation diagnosis has also shown in many cases an extra effective measure in reducing the birth of TM children[42]. However, it is a more difficult and risky procedure than prenatal diagnosis with low success rate and is also more expensive. The prospects of use of *in utero* allogeneic bone marrow transplantation in the future may decrease further the birth of TM children but this still requires further research[60,133].

The treatment of TM patients is also entering a new phase. Bone marrow transplantation offers a cure for TM patients but the risk of rejection of the transplant and of fatalities is still high[57-60]. The vast majority of TM patients follow the treatment of regular red blood cell transfusion and chelation therapy. Complete treatment of iron overload in TM patients is now envisaged from the use of the ICOC L1/DF combination protocol, which is leading progressively to the elimination of all excess toxic iron load and the attainment of normal iron levels, which are within the physiological range. This outcome increases the prospects of living free of iron related toxicity complications and with longer-term survival, similar to that observed in the general normal population[110,134].

In addition to the increased survival, the quality of life of TM patients has drastically changed in Cyprus and other countries adopting similar policies. The improvement in treatment, which mainly involved the introduction of L1 and other advances such as the introduction of elastomeric pumps for the delivery of subcutaneous DF and also the set up of a specialist multi-disciplinary team for the clinical follow up of TM patients has also contributed to this transition. For example, some of the TM patients have been attending tertiary education and many are employed in different professional sectors. In addition the marital and family status of the TM patients has also changed and most adults are married and have children. For example, in a fertility report of Cypriot TM women in 2004, improved attainment of reproductive capacity has been observed with a total of 358 successful pregnancies[134]. An increasing number of TM patients are now over 50 years of age and have become grandparents. This development followed the introduction of L1 and the L1/DF combination, where there was a significant drop in the mortality rate of TM patients from cardiac complications and also the reversal of iron overload toxicity in other organs[9,25-29,113,134].

The apparent increase in survival of TM patients is observed in Cyprus, and also many other countries using related chelation therapy and similar supportive therapy protocols[25-29]. The transition leading to the longer term survival outcome appears to be related to the improved compliance in the oral chelation therapy and more importantly to the ability of L1 to mobilise rapidly excess iron deposits from the heart, which has also been repeatedly confirmed in several thalassaemia centers using the T2* and T2 relaxation time MRI techniques[54,55,111,116].

The ability of L1 to reverse cardiac, liver, kidney and possibly other organ or tissue iron related damage in dif-

ferent categories of patients and its antioxidant properties provide further evidence of its potent therapeutic effects[69,70]. This therapeutic option may also apply to FA patients at present where so far no effective treatment has become available and L1 appears to offer the most promising therapeutic option, especially if used at the appropriate doses and protocols.

The exceptional therapeutic properties of L1 by comparison to other therapeutics in FA include several advantages such as its ability to enter most tissues and organs including the heart and the brain and also subcellular organelles such as mitochondria and cytosolic compartments at high therapeutic concentrations and to remove progressively and effectively toxic iron deposits. The efficacy of L1 in targeting iron toxicity arising from labile toxic iron forms and also from gross body or focal localised iron deposits, as well as its ability to redistribute iron *via* transferrin and reach iron balance in tissues, can make L1 a universal iron detoxifier and iron metabolic regulatory drug in many diseases including FA[64,69,117,135,136]. In addition, L1 can also act as an antioxidant by inhibiting iron catalysed free radical reactions and cascades arising from mitochondrial malfunction, which is considered a major cause of toxicity in the pathology of FA and also of other diseases (Figure 2)[5,69,70].

There are many other therapeutic advantages in the clinical use of L1 in FA and other conditions[64,69,70,137]. For example, the role of other metals in addition to iron in the cause and acceleration of neurodegeneration in FA and also other diseases is an expanding area under investigation[138-141]. Within this context, L1 can chelate and mobilise aluminium and copper and eliminate their associated toxicity as shown in cell and animal studies and also in clinical conditions[64,142-144].

Future prospects regarding the treatment of FA include the identification of all the molecular targets associated with the pathological mechanisms, the design of new therapeutics and protocols, as well as the optimisation of the existing experimental treatments. Within this context the importance of mitochondrial and cytosolic iron deposition and its significance in the neuropathy, cardiomyopahy as well as other organ damage needs to be further investigated, qualified, quantified and the associated toxicity defined similar to the iron overload toxicity in TM[54-56].

The optimisation of chelation therapy in FA needs to be further investigated. More effective removal of iron in FA can be achieved using higher doses of L1 than those used in the reported clinical trials. In this case two or three divided doses to a total of 50-75 mg/kg per day of L1 can be used depending on the level of focal iron deposition, which can be determined by the MRI T2 and T2* techniques[54-56]. Similarly, prophylactic use of lower doses of L1 (*e.g.*, 10 mg/kg per day) can also be used at the early stages in young diagnosed patients to delay or prevent focal iron load deposition and the onset of pathological symptoms.

Alternative and complimentary experimental treatments to that of iron chelation therapy could also be considered. For example, regular venesection treatment at an early age can be investigated in FA patients not only for prophylaxis but also as a form of main treatment at later stages. This treatment may be more appropriate for FA patients with excess mitochondrial and cytosolic iron deposition. Chronic venesection treatment is simple and inexpensive and can be well tolerated and used before and after the onset of the symptoms in FA patients. The rate of venesection can vary from that of regular blood donors to a more intensive one similar to that of hereditary haemochromatosis patients[13]. Reduction in the rate of organ and mitochondrial focal iron load deposition and associated toxicity as well as delay in the onset of pathological symptoms is expected using this form of treatment.

The therapeutic use of hyperbaric oxygen as a prophylactic measure in asymptomatic FA patients before the onset of mitochondrial focal iron load could also be considered for improvement of neuronal function[145]. Relative improvements were previously observed following the therapeutic use of hyperbaric oxygen in some stroke patients who had focal neuronal damage and substantial reduction of ATP production[145]. This method can also be applied to FA patients with focal iron deposits and pathological symptoms but special safety procedures may be required because of possible exacerbation of the mitochondrial induced oxidative stress. Combination therapies of chelating drugs, antioxidants, hyperbaric oxygen and venesection could provide an alternative investigational approach to the treatment of FA patients.

Gene therapy is the ultimate target for the complete treatment of both TM and FA[146,147]. However, such experimental therapies have been tried in many diseases with no optimistic outcome so far[146,147]. In all cases of the proposed introduction of new protocols or new treatments for FA or TM patients, a risk/benefit assessment is necessary for comparison of the existing and the new treatments. This assessment is particularly important for FA patients where no effective treatments are available at present.

The recent progress in the prevention, diagnosis and treatment of TM and FA patients in Cyprus can serve as a model by other countries and worldwide organisations such as the World Health Organization for minimising the births of TM and FA children and for improving the treatment of these and other inherited diseases worldwide. In particular, the organisational health structures adopted and the new treatments provided are shifting the disease profile of TM from a fatal disease to chronic one, in Cyprus and other countries using similar models. It is also hoped that the same model could be adopted for FA, including the introduction of effective new treatments.

Further efforts are needed for the development and improvement of organisational health structures for the prevention, diagnosis and treatment of TM and FA patients in developing countries, where health resources are extremely limited. Similarly, further advances in the de-

sign of personalised medicine which can provide optimal therapies for individual patients are also in progress and are the subject of in depth investigation.

CONCLUSION

The experience of the Cypriot model of organisational health structure implies that prenatal and antenatal diagnoses can be successfully used for reducing substantially the birth rate of TM and FA patients and the overall prevention of the incidence of these inherited pathological diseases worldwide. The introduction of new treatment methods, particularly the use of effective therapeutic protocols of L1, resulted in many improvements including better compliance with chelation therapy and improved the quality of life, as well as reduction or reversal of iron overload related organ damage and an overall increase in the survival of the TM patients in Cyprus and other countries using similar iron chelation protocols.

Similarly, the introduction of L1 in the treatment of FA patients increases the prospects of reducing the morbidity and mortality rates observed in this disease. New possible treatments for FA patients such as improved chelation dose protocols of L1, venesection and hyperbaric oxygen could be investigated and selected on individual patient cases based on a risk/benefit assessment. Improved diagnostic techniques for the detection of stored iron such as MRI and of iron toxicity could improve the therapeutic targeting methods for both TM and FA.

It appears that recent advances in the diagnosis of the pathogenesis and the treatment are changing TM and FA from fatal to chronic diseases.

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ORIGINAL ARTICLE

Methodical and pre-analytical characteristics of a multiplex cancer biomarker immunoassay

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Abstract

AIM: To test the methodical and pre-analytical performance of a new multiplex cancer biomarker panel using magnetic beads.

METHODS: The MILLIPLEX® MAP Human Circulating Cancer Biomarker Magnetic Bead Panel 1 comprises the tumor markers carcinoembryonic antigen, alpha-fetoprotein, total prostate-specific antigen, cancer antigen 15-3, cancer antigen 19-9, cancer antigen 125, cytokeratine 19-fragment, β-human chorionic gonadotropin, human epididymis protein 4, osteopontin, prolactin, the cell death and angiogenesis markers soluble Fas, soluble Fas-ligand, tumor necrosis factor related apoptosis-inducing ligand, vascular endothelial growth factor and the immunological markers interleukin-6 (IL-6), IL-8, tumor necrosis factor-α, transforming growth factor α, fibroblast growth factor-2, macrophage migration inhibitory factor, leptin, hepatocyte growth factor, and stem cell factor. We determined intra- and inter-assay imprecision as well as dilution linearity using quality controls and serum pools. Furthermore, the stability of the 24 biomarkers examined in this panel was ascertained by testing the influence of different storage temperatures and time span before centrifugation.

RESULTS: For all markers measured in the synthetic internal quality controls, the intra-assay imprecision ranged between 2.26% and 9.41%, while for 20 of 24 measured markers in the physiological serum pools, it ranged between 1.68% and 12.87%. The inter-assay imprecision ranged between 1.48%-17.12% for 23 biomarkers in synthetic, and between 4.59%-23.88% for 18 biomarkers in physiological quality controls. Here, single markers with very low concentration levels had increased imprecision rates. Dilution linearity was acceptable (70%-130% recovery) for 20 biomarkers. Regarding pre-analytical influencing factors, most markers were stable if blood centrifugation was delayed or if serum was stored for up to 24 h at 4 ℃ and 25 ℃ after centrifugation. Comparable results were obtained in serum and plasma for most markers. However, great changes were observed for single markers.

CONCLUSION: MILLIPLEX® MAP Human Circulating Cancer Biomarker Magnetic Bead Panel 1 assay is a stable and precise method for detection of most biomarkers included in the kit. However, single markers have to be interpreted with care.

Key words: Multiplex immunoassay; Tumor marker; Cytokines; Cell death markers; Methodical evaluation

Core tip: In this study, the methodological quality of a new research-use-only multiplex magnetic bead assay,

particularly designed for cancer diagnosis, was evaluated. This attractive panel includes 24 biomarkers: established as well as auspicious tumor markers and markers deriving from the fields of apoptosis, immunology and angiogenesis. Herewith, the complexity and multifactorial background of a cancer disease is depicted. Measurements were performed with physiological serum pools and intra- and inter-assay imprecision as well as dilution linearity were assessed. Furthermore, the influence of preanalytical factors was investigated.

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INTRODUCTION

Despite of essential achievements in cancer research concerning diagnosis, therapy options and follow up methods, cancer diseases still present a global health problem[1]. A great variety of clinical and imaging tools are applied to diagnose tumor masses and screening programs have been established for certain entities[2]. Some serum tumor markers, such as alpha-fetoprotein (AFP), cancer antigen 125 (CA 125), CA 15-3, CA 19-9, carcinoembryonic antigen (CEA) or prostate-specific antigen (PSA), have been introduced as supplementary diagnostic tools, but none of the above is recommended as a singular method to define a cancer diagnosis[3-5].

Cancer is nowadays perceived as a complex disease involving inflammatory and immunological systems and programs of cell death[6,7]. Thus, the diagnostic opportunity could be greatly enhanced by measurement of more than one marker as a fraction of information required to understand a complex pathological state[8,9]. Based on these findings, methods for parallel tumor marker testing have become more and more interesting in cancer research. Here, biomarkers, representing different systemic processes, such as inflammation, angiogenesis or cell death, can be combined with established tumor markers in one panel and potentially increase diagnostic accuracy[10-12].

Multiplex based immunoassays belong to the leading methods in this field. They are based on flow cytometry principles applied to labeled microspheres and depict an "ELISA on a bead"[13]. They offer several advantages, such as high-throughput performance, low material requirement, wide range application and cost- and time-effective multiplexing of more than 20 parameters[8,13].

However, the implementation of bead based multiplex assays has not yet been established in clinical routine[14]. Currently used tumor markers are mainly tested with single parameter assays. Not least due to the great potential of differently composed assays or marker panels, respectively, this field requires further research in order to assess assay quality, increase comparability of multiplex assays, and to encourage consistent guidelines which as of yet are non-existent[3,9,15].

As already shown by other research groups marker combination has the potential to greatly improve the quality of early diagnosis and other therapeutically relevant applications[12,16,17]. Several manufacturers offer diverse panels of markers, mainly for the combined measurement of many immunological and metabolic markers. For oncological purposes, the MILLIPLEX® Map Human Circulating Cancer Biomarker Magnetic Bead Panel Kit (EMD Millipore) was recently released. It represents an attractive option particularly for study settings. This kit includes reagents for the detection of 24 biomarkers, which portray a widespread spectrum of already validated as well as upcoming auspicious oncological, cell death, angiogenesis and immunological biomarkers, such as CEA, AFP, total prostate-specific antigen (total-PSA), CA 15-3, CA 19-9, CA 125, cytokeratine 19-fragment (CYFRA 21-1), β-human chorionic gonadotropin (β-HCG), human epididymis protein 4 (HE4), osteopontin (OPN), prolactin, soluble Fas (sFas), soluble Fas-ligand (sFasL), tumor necrosis factor related apoptosis-inducing ligand (TRAIL), vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), IL-8, tumor necrosis factor-α (TNFα), transforming growth factor α (TGFα), fibroblast growth factor-2 (FGF2), macrophage migration inhibitory factor (MIF), leptin, hepatocyte growth factor (HGF) and stem cell factor (SCF).

However, in order to be used in studies and for clinical measurements, this panel must fulfill certain requirements, such as high reliability, accuracy, robustness as well as high analytical and clinical sensitivity and specificity[18,19]. Furthermore, the analytes must be stable against potentially influencing pre-analytical factors[3]. This study was carried out to critically test whether all or only some of the markers fulfill these basic methodical quality criteria and can thus be recommended for application in clinical or study conditions.

MATERIALS AND METHODS

In order to assess the methodological performance of MILLIPLEX® MAP Human Circulating Cancer Biomarker Magnetic Bead Panel 1, 96 Well Plate Assay we tested intra- and inter-assay imprecision as well as dilution linearity.

Standard samples plus quality controls QC 1 and QC 2 delivered by the kits were used for the internal methodological control. Standard 7 depicted the basis for a dilution line with the factor 1:3 from high to lower biomarker concentrations. The standard dilution line as well as the concentrations of QC 1 and QC 2 were predefined by the manufacturer.

For external control, we produced two serum pools with levels in the moderate to high and in the very low value range for most markers (pool 1 and pool 2). To create pool 1, 37 residual and anonymized sera of daily

clinical routine diagnostics were combined. Inclusion criteria were present high levels of the inflammation parameter C-reactive protein and well above average levels of the biomarkers AFP, β-HCG, CA 15-3, CA 125, CA 19-9, CEA, NSE and PSA. Here, patient history was not considered. Pool 2 is a combination of two sera taken from young healthy women (mean age 23.5 years). The sera pools, standard samples and quality controls QC 1 and QC 2 were run in duplicate as minimum within each plate.

In order to evaluate the linearity of dilution, a 50% dilution of the higher concentrated pool 1 was prepared by mixing the pool 1 sample with the appropriate amount of serum matrix enclosed in our kit. We defined the acceptable range for the recoveries as values between 70% and 130%.

Next, the estimation of possible affecting pre-analytical issues was tested. Briefly, samples of two different patients were stored at 25 ℃ (room temperature) for 6 and 24 h prior to centrifugation and subsequent freezing at -80 ℃. In a further experiment, samples were stored at 4 ℃ and 25 ℃ for 6, 24 and 48 h, respectively, after centrifugation and before freezing at -80 ℃. As reference control we used the corresponding samples, which were directly frozen after centrifugation. Finally, biomarkers were tested in serum and EDTA-plasma samples that were taken in parallel from the two healthy donors. All different conditions were measured in a single plate at a later time point to avoid inter-assay interferences.

MILLIPLEX® MAP Kit on the Bio-Plex® 200 System

The MILLIPLEX® MAP Human Circulating Cancer Biomarker Magnetic Bead Panel 1, 96 well plate assay purchased from EMD Millipore included all the reagents as well as an appropriate plate required by the assay. The procedure was conducted by experienced staff according to the manufacturer's protocol. For washing steps, the Bio-Plex® Pro Ⅱ wash station was applied. All plates were run on the Bio-Plex® 200 System. Before each assay run, the system was calibrated with the Bio-Plex® calibration kit and validated with the Bio-Plex® validation kit 4.0. Bio-Plex® sheath fluid served as the delivery medium for the samples. Analysis was performed with Bio-Plex® manager 6.1. Within the device settings, 50 events per bead region were defined as minimum criterion.

Principle

MILLIPLEX® MAP Kit Human Circulating Cancer Biomarker Magnetic Bead Panel 1 was developed as an immunoassay on the surface of fluorescent-coded magnetic beads (MagPlex™-C microspheres). The proportion of two fluorescent dyes on these beads forms the code and determines in such way up to 100 different kinds of beads. Here, we have 24 differently coded bead groups, each of which is coated with a specific capture antibody to detect one of the 24 biomarkers which are CEA, AFP, PSA, CA 15-3, CA 19-9, CA 125, CYFRA 21-1, β-HCG, HE4, osteopontin and prolactin, the cell death and angiogenesis markers sFas, sFasL, TRAIL and VEGF as well as the immunological markers IL-6, IL-8, TNFα, TGFα, FGF-2, MIF, leptin, HGF and SCF.

The binding of specific analytes begins in the bead mixture suspended with a test sample. Next, a biotinylated detection antibody is introduced and subsequent incubation with streptavidin-phycoerythrin (PE) conjugate is performed to complete the reaction on the microspheres.

Finally, the assay is analyzed by the Bio-Plex® 200 system. Here, the beads coupled with the capture antibody bound to the specific analyte, biotinylated detection antibody and streptavidin-PE on its surfaces pass through a laser, which excites the internal dyes. A second laser excites the signal of PE. High-speed digital-signal processors identify the beads and detect the fluorescent signal intensity in order to quantify the assay result.

Procedures

All reagents and sera were brought to room temperature before use. Wash buffer, assay buffer, serum matrix, standard 7, quality controls 1 and 2, beads, detection antibodies, and streptavidin-PE were prepared as recommended by the manufacturer. Serum samples were thawed and individually vortexed for 15 s. Thereafter, they were centrifuged (Eppendorf centrifuge 581OR) at 3500 rpm for one min. Next, 15 μL of sample was mixed with 75 μL of serum matrix creating a 1:6 dilution. To create a homogeneous mixture of all antibody conjugated beads, every vial containing one set of microspheres was sonicated for 30 s and then vortexed for one minute. One hundred and fifty microliter of each vial was transferred into a mixing bottle that was vortexed again for one minute. The beads were protected from exposure to light throughout the assay. For pre-wetting, 200 μL assay buffer was pipetted into each well, the plate was covered with a sealer and then shaken at 700 rpm for 10 min. The fluid was removed by tapping the plate on a paper towel and centrifuging it briefly at 3500 rpm lying top down on a paper sheet in the centrifuge. Twenty-five microliter of background, standard 1-7 and quality controls 1 and 2 were pipetted in duplicate into the appropriate wells and 25 μL of serum matrix was added. Next, sample wells were filled with 25 μL of assay buffer and 25 μL of the diluted sera was pipetted into the appropriate wells. Finally, the magnetic bead mixture was vortexed for 1 min and 25 μL were pipetted into each well. The plate was sealed, covered with aluminum foil and then shaken at 700 rpm for 16 h at 4 ℃. After incubation time the magnetic bead plates were washed as recommended by the method protocol three times using assay buffer by means of Bio-Plex® Pro Ⅱ wash station. Then 25 μL of detection antibodies were added to each well, the plate was sealed and covered with aluminum foil and shaken at 700 rpm for 1 h. Now 25 μL of streptavidin-PE per well were added and the plate was again sealed, covered with aluminum foil and shaken at 700 rpm for 30 min. Thereon the three washing steps were performed as described above. One hundred microliter of sheath fluid

were pipetted into each well, the plate sealed and covered with aluminum foil and shaken at 700 rpm for 5 min in order to resuspend the beads. Lastly, the plates were run on the Bio-Plex® 200 system.

Expected concentrations of each tested biomarker for standards 1-7 were entered into the system prior to running the assay. The device detects appropriate fluorescence intensities (FI) and creates a standard curve for each marker. These curves are generated by linking the measured FI values with the expected concentration of markers in standards 1-7. Further translation of FI-values in concentration levels of all the following samples is based on these curves. Depending on the five parameter logistic, the function possesses predefined points of accepted extrapolation, which are the minimum and maximum asymptotes. For the lower limits in our study, we accepted an extrapolation in round terms in the middle between the lowest standard and the minimum asymptote point. Due to the phenomenon of heteroscedasticity towards higher concentrations, here, the accepted extrapolation was defined as an approximation of the highest standard value. These limits consequently illustrate our measuring range.

As all physiological serum samples were diluted 1:6 with serum matrix included into the kit, the dilution factor 6 was considered by the software before yielding the final concentration of the samples. For convenience of comparability in added tables, we multiplied all non diluted concentrations (accepted measuring range, observed concentration of QC 1, QC 2 and standard 5) with the factor 6.

The results from the software of Bio-Plex® 200 contain the measured FI and when duplicates were run, also the corresponding means, standard deviation and coefficients of variation (CV in %) as well as the corresponding concentrations.

Statistical analysis

In order to assess intra- and inter-assay imprecision FI-based CVs were used. We also determined the CVs based on the observed concentration for the analysis. Means and ranges were calculated for all comparisons. In order to quantify the dilution linearity, we determined observed concentration-based recoveries related to the corresponding expected values for the 50% dilution of pool 1. The evaluation of the pre-analytical influence of different storage conditions is represented by calculated recoveries based on FI results.

All in all, we tested ten kits or rather ten plates. The first five plates were ordered as a batch and were measured subsequently and strictly under the same conditions. The following five plates were of the same lot, but were ordered and measured about six months later under different conditions. Therefore, the main method evaluation in our study is based on the first five kits. However, results of the overall evaluation are also shown. In one assay, a pipetting error of the pool samples occurred and the respective values were omitted.

RESULTS

Intra-assay imprecision

The intra-assay imprecision as the mean CV (in %) over all plates for synthetic quality controls QC 1, QC 2 and standard 5 as well as for physiological serum pools 1 and 2 was calculated for five different magnetic plates relating to each tested biomarker. Here, only results within the measuring range were included.

In QC 1 and QC 2, 22 and 24 markers had an FI-based CV below 10%. In QC 2, 21 biomarkers were measured with a CV less than 5%. The CVs ranged between 3.81% (AFP) and 13.38% (FGF2) for QC 1 and between 2.06% (total PSA) and 5.56% (β-HCG) for QC 2. Observed concentration-based CVs ranged between 4.23% (AFP) and 9.41% (FGF2) for QC 1 and between 2.26% (TRAIL) and 7.69% (CA 19-9) for QC 2 (Table 1).

In the standard 5-sample, all of the 24 biomarkers were measured with CV values below 5%. The range was between 0.91% (OPN)-4.41% (VEGF).

In the physiological serum pool 1, all biomarkers showed an FI-based CV below 10%, while 13 biomarkers had a CV below 5%. The values ranged from 1.89% (MIF) to 8.71% (FGF2). Observed concentration-based CVs ranged from 1.68% (MIF) to 36.09% (β-HCG) with 12 biomarkers measured with a CV below 5% and four biomarkers exceeding the 10% range (Table 1).

In physiological serum pool 2, FI-based imprecision ranged from 1.65% (CA 19-9) to 14.31% (MIF) with only one CV (MIF) found to be higher than 10%. CVs of 11 biomarkers fell below 5%. Observed concentration-based imprecision ranged from 1.47% (sFas)-15.66% (MIF) with seven biomarkers measured with a CV below 5% and seven biomarkers exceeding the 10% range (Table 1).

When intra-assay imprecision was evaluated for all ten plates, the CVs were somewhat higher for pools and QC samples (Table 2).

Inter-assay imprecision

FI-based imprecision: The inter-assay imprecision was performed by calculating the CV in % involving all fluorescence intensity results for QC 1, QC 2, standard 5, pool 1 and pool 2 over five different magnetic plates relating to each tested marker. Here, only results within the measuring range were included.

For synthetic QC 1, QC 2 and standard 5, 16, 17 and 18 markers had imprecision below 20%, ranging between 8.85% (IL-8) and 45.75% (OPN), 8.88% (CEA) and 29.04% (OPN) as well as between 8.61% (total PSA) and 42.71% (CYFRA 21-1) (Table 3).

In the higher-concentrated physiological serum pool 1, the inter-assay imprecision fell below 20% for 18 biomarkers with the total range between 4.49% (β-HCG) and 46.72% (OPN). In the very low serum pool 2, only four biomarkers were measured with a CV below 20%, collectively ranging between 10.19% (HGF) and 85.54% (OPN) (Table 3).

Table 1 Intra-assay imprecision

Biomarker	Measuring range	Unit	QC 1			QC 2			St 5			Pool 1			Pool 2		
			Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%
CEA	100-120000	pg/mL	2505	7.24	8.87	10933	2.32	3.39	2150	1.29		38897	2.29	3.98	466	6.77	7.78
AFP	500-600000	pg/mL	11980	3.81	4.23	54865	2.4	3.29	10703	2.88		455084	3.46	12.87	1598	4.99	11.99
Total PSA	50-60000	pg/mL	1230	5.97	7.9	5880	2.06	3.41	1057	1.92		7762	2.46	4.16	< MR	< MR	< MR
CA 15-3	0.5-600	U/mL	12	9.1	8.43	57	4.06	4.5	11	3.05		29	6.48	6.35	24	3.87	3.7
CA 19-9	2-3000	U/mL	61	9.54	7.35	292	5.27	7.69	54	3.2		33	3.35	2.66	10	1.65	1.75
CA 125	2-4000	U/mL	71	6.77	6.68	347	4.14	4.4	68	2.27		302	4.15	4.36	5.53	6.23	11.25
β-HCG	0.2-400	mU/mL	8	8.86	5.54	39	5.56	4.25	7	1.54		0.65	6.37	36.09	< MR	< MR	< MR
CYFRA 21-1	500-900000	pg/mL	29454	7.92	6.98	155151	3.7	3.18	16614	1.52		9724	4.22	4.77	3506	8.07	13.12
HE4	2000-3000000	pg/mL	55648	8.73	6.98	291745	5.17	5.23	55123	4.1		2276	8.16	9.58	< MR	< MR	< MR
Prolactin	500-600000	pg/mL	11868	5.69	4.73	60195	3.04	3.55	10922	2.61		12057	4.37	3.69	10765	3.08	2.52
Leptin	500-600000	pg/mL	13056	8.97	5.05	62955	2.75	2.38	10971	1.94		13430	7.17	4.04	8468	2.78	1.48
OPN	2000-3000000	pg/mL	53895	8.94	7.32	236975	3.72	6.02	43171	0.91		73954	4.46	4.01	10870	2.75	2.25
HGF	100-120000	pg/mL	2452	9.76	8.64	12766	4.06	4.28	2154	2.86		785	3.11	2.86	283	5.65	7.44
MIF	100-120000	pg/mL	2230	7.83	5.97	11061	4.5	4.45	2209	3.06		574	1.89	1.68	513	14.31	15.66
sFas	100-150000	pg/mL	2486	6.3	5.53	12705	3.19	2.77	2779	2.4		3548	4.52	3.89	2217	1.67	1.47
sFasL	50-60000	pg/mL	1219	5.65	5.71	5898	2.17	2.28	1091	2.86		< MR	< MR	< MR	< MR	< MR	< MR
TRAIL	10-12000	pg/mL	252	7.23	7.41	1233	2.16	2.26	221	2.49		65	3.4	3.62	92	3.32	3.5
VEGF	50-60000	pg/mL	1127	13.04	8.8	6110	3.97	2.78	1115	4.41		< MR	< MR	< MR	< MR	< MR	< MR
IL-6	2-3000	pg/mL	49	9.2	9.27	226	4.33	4.21	53	3.43		16	5.66	7.02	< MR	< MR	< MR
IL-8	5-6000	pg/mL	122	5.53	5.67	605	2.19	2.34	110	1.71		33	5.85	6.7	6.71	4.49	9.43
TNFα	5-6000	pg/mL	114	7.77	7.97	562	2.88	3.17	110	2.25		13	4.56	5.66	6.41	3.12	6.68
TGFα	10-12000	pg/mL	237	6.36	6.29	1180	3.02	3.11	221	1.23		24	5.17	8.75	18	4.49	11.59
FGF2	50-50000	pg/mL	1101	13.38	9.41	5571	2.61	2.85	1066	3.08		55	8.71	18.11	79	9.57	14.52
SCF	20-30000	pg/mL	599	8.41	7.97	2813	3.02	2.88	550	1.17		58	6.99	10.97	50	8.32	13.4

Intra-assay imprecision results for all 24 tested markers based on FI as well as observed concentration (Obs Conc) results of assays 1-5 for synthetic quality controls QC 1, QC 2, standard 5 as well as for physiological serum pools 1 and 2. AFP: Alpha-fetoprotein; CA 125: Cancer antigen 125; CEA: Carcinoembryonic antigen; CV: Coefficient of variation; CYFRA 21-1: Cytokeratine 19-fragment; FGF2: Fibroblast growth factor-2; FI: Fluorescent intensity; HE4: Human epididymis protein 4; HGF: Hepatocyte growth factor; IL-6: Interleukin-6; MIF: Macrophage migration inhibitory factor; MR: Measuring range; Conc: Concentration; OPN: Osteopontin; QC 1: Quality control 1; SCF: Stem cell factor; sFas: Soluble Fas; sFasL: Soluble Fas-ligand; TGFα: Transforming growth factor-α; TNFα: Tumor necrosis factor-α; total PSA: Total prostate-specific antigen; TRAIL: Tumor necrosis factor related apoptosis-inducing ligand; VEGF: Vascular endothelial growth factor; β-HCG: β-human chorionic gonadotropin.

Observed concentration based imprecision: In synthetic internal controls, the imprecision was below 20% for 23 biomarkers in QC 1 and QC 2. The same applied to 24 biomarkers in standard 5. The corresponding ranges were 2.44% (Leptin) to 27.37% (CYFRA 21-1), 1.48% (β-HCG) to 40.96% (CYFRA 21-1) and 0.32% (total PSA) to 4.15% (CEA) (Table 3).

In the physiological serum pool 1, the imprecision fell below 20% for 16 biomarkers and values ranged from 4.59% (total PSA) to 68.81% (FGF2). Notably, single markers in pool 1 with very low concentration levels had considerably higher imprecision rates. In serum pool 2 with very low values for all markers, only one marker (CEA) was measured with a CV less than 20%. Here, the imprecision ranged in total between 14.43% (CEA)-66.04% (AFP) (Table 3).

When inter-assay imprecision was evaluated for all ten plates, the CVs were somewhat higher for pools and QC samples (Table 4).

Table 2 Extended intra-assay imprecision

Biomarker	Measuring range	Unit	QC 1			QC 2			St 5			Pool 1			Pool 2		
			Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%
CEA	100-120000	pg/mL	2545	8.32	11.34	12727	7.61	12.23	2146	4.11		39391	2.1	3.63	560	8.09	5.67
AFP	500-600000	pg/mL	12038	6.09	6.9	56960	3.08	4.23	10754	4.45		493993	2.39	8.73	1489	6.87	11.8
Total PSA	50-60000	pg/mL	1421	5.76	7.78	6172	3.75	6.27	1052	4.1		8246	1.59	2.69	68	9.76	7.02
CA 15-3	0.5-600	U/mL	12	9	8.7	62	4.18	4.96	11	4.32		33	5.31	5.23	26	9.19	8.66
CA 19-9	2-3000	U/mL	64	10.63	8.63	329	4.83	7.1	54	5.05		36	2.9	2.39	10	4.87	6.17
CA 125	2-4000	U/mL	74	7.02	7.03	370	3.61	3.78	68	2.84		317	3.59	3.64	6.13	4.74	8.54
β-HCG	0.2-400	mU/mL	7.85	10.62	7.02	42	4.89	3.67	7	5.08		1.2	6.76	18.28	0.55	8.17	31.39
CYFRA 21-1	500-900000	pg/mL	24985	7.62	8.22	152731	12.13	4.28	16601	3.32		8627	4.34	5.73	3506	8.33	13.12
HE4	2000-3000000	pg/mL	55766	11.42	9.39	295050	4.28	4.45	55043	1.9		2288	6.47	7.67	2590	11.67	8.86
Prolactin	500-600000	pg/mL	11952	7.86	6.96	60823	3.43	4.13	10863	4.49		12191	3.31	2.83	10602	6.1	4.71
Leptin	500-600000	pg/mL	13001	12.79	7.06	64533	2.79	2.4	11020	5.73		14622	5.67	3.18	9145	6.58	3.4
OPN	2000-3000000	pg/mL	57576	9.61	8.58	240688	4.93	9.29	42845	3.77		75488	3.61	3.35	10700	5.59	4.85
HGF	100-120000	pg/mL	2546	9.92	8.68	13471	3.23	3.3	2171	2.51		894	2.82	2.53	436	7.6	8.8
MIF	100-120000	pg/mL	2263	10.84	10.06	12332	5.12	5.99	2220	5.19		876	2.06	1.86	517	11.4	17.25
sFas	100-150000	pg/mL	2533	7.15	6.72	13212	3.91	3.47	2775	3.94		4005	3.54	3.07	2472	4.88	4.34
sFasL	50-60000	pg/mL	1245	6.35	6.49	6023	2.9	3.15	1093	5.13		< MR	< MR	< MR	< MR	< MR	< MR
TRAIL	10-12000	pg/mL	253	8.52	8.64	1269	2.65	2.72	220	3.73		72	4.22	4.71	98	6.19	6.44
VEGF	50-60000	pg/mL	1146	16.04	11.12	6155	4.61	3.56	1100	2.88		< MR	< MR	< MR	<MR	< MR	< MR
IL-6	2-3000	pg/mL	54	11.72	12.01	268	6.35	6.11	54	1.79		21	6.25	7.44	< MR	< MR	< MR
IL-8	5-6000	pg/mL	124	7.8	9.02	668	6.56	9.49	111	3.94		26	5.05	9.13	6.70	6.22	9.43
TNFα	5-6000	pg/mL	117	10.43	10.35	583	3.68	3.8	111	4.86		16	5.27	9.14	7.85	5.41	4.61
TGFα	10-12000	pg/mL	238	8.86	8.94	1210	3.78	4.05	221	4.91		27	3.99	9.09	18	5.32	9.69
FGF2	50-50000	pg/mL	1104	20.18	14.17	5708	2.97	3.07	1068	2.04		68	9.63	13.35	80	7.72	7.97
SCF	20-30000	pg/mL	603	8.04	7.66	2951	4.39	4.32	549	3.96		79	6.49	8.93	59	8.5	15.12

Intra-assay imprecision results for all 24 tested markers based on FI as well as observed concentration (Obs Conc) results of all assays 1-10 for synthetic quality controls QC 1, QC 2, standard 5 as well as for physiological serum pools 1 and 2. AFP: Alpha-fetoprotein; CA 125: Cancer antigen 125; CEA: Carcinoembryonic antigen; CV: Coefficient of variation; CYFRA 21-1: Cytokeratine 19-fragment; FGF2: Fibroblast growth factor-2; FI: Fluorescent intensity; HE4: Human epididymis protein 4; HGF: Hepatocyte growth factor; IL-6: Interleukin-6; MIF: Macrophage migration inhibitory factor; MR: Measuring range; Conc: Concentration; OPN: Osteopontin; QC 1: Quality control 1; SCF: Stem cell factor; sFas: Soluble Fas; sFasL: Soluble Fas-ligand; TGFα: Transforming growth factor-α; TNFα: Tumor necrosis factor-α; total PSA: Total prostate-specific antigen; TRAIL: Tumor necrosis factor related apoptosis-inducing ligand; VEGF: Vascular endothelial growth factor; β-HCG: β-human chorionic gonadotropin.

Dilution linearity

The 50% dilutions of pool 1 samples were run in each of the five plates. The dilution of 20 biomarkers fell into the accepted recovery of 70%-130%. Range of all markers was between 53.19% (β-HCG) and 136.24% (FGF2) (Figure 1). Here, concentration levels calculated by extrapolation were included. VEGF was the only biomarker without any calculable levels of concentration.

Different storage conditions

Samples centrifuged and measured after being stored for 6 h at room temperature (25 ℃) yielded a median recovery of 95.0% [range: 84.5% (IL-6)-204.4% (MIF)], while a centrifugation after 24 h showed a stronger effect on some biomarkers with a median recovery of 108.2% ranging from 92.8% for TRAIL to 1453.3% for IL-8. However, only two biomarkers after 6 h and three biomarkers after 24 h failed the accepted range of recovery (IL-8 and MIF) (Figure 2).

Table 3 Inter-assay imprecision

Biomarker	Measuring range	Unit	QC 1			QC 2			St 5			Pool 1			Pool 2		
			Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%
CEA	100-120000	pg/mL	2505	10.45	3.92	10933	8.88	7.62	2150	11.53	4.15	38897	4.53	6.58	466	15.14	14.43
AFP	500-600000	pg/mL	11980	13.92	5.32	54865	12.72	5.09	10703	12.14	3.43	455084	8.59	20.68	1598	34	66.04
Total PSA	50-60000	pg/mL	1230	15.82	5.11	5880	11.51	11.21	1057	8.61	0.32	7762	7.88	4.59	< MR	< MR	< MR
CA 15-3	0.5-600	U/mL	12	18.57	6.38	57	21.29	2.85	11	20.52	1.79	29	16.48	8.48	24	27.78	21.2
CA 19-9	2-3000	U/mL	61	20.3	4.79	292	12.19	4.58	54	11.44	2.19	33	17.49	7.29	10	19.24	23.19
CA 125	2-4000	U/mL	71	13.39	3.23	347	11.19	3.9	68	11.73	1.91	302	6.26	8.27	5.53	22.25	36.3
β-HCG	0.2-400	mU/mL	8	17.8	4.39	39	16.3	1.48	7	15.48	2.41	0.65	4.49	61.53	< MR	< MR	< MR
CYFRA 21-1	500-900000	pg/mL	29454	16.78	27.37	155151	15.64	40.96	16614	42.71	1.14	9724	38.02	50	3506	21.25	30.8
HE4	2000-3000000	pg/mL	55648	27.52	5.48	291745	23.45	3.46	55123	23.76	2.74	2276	14.67	63.85	< MR	< MR	< MR
Prolactin	500-600000	pg/mL	11868	26.35	4.16	60195	15.98	2.69	10922	14.69	1.97	12057	21.22	10.08	10765	35.99	20.91
Leptin	500-600000	pg/mL	13056	23.59	2.44	62955	14.97	3.92	10971	15.63	1.81	13430	18.98	8.79	8468	36.1	23.77
OPN	2000-3000000	pg/mL	53895	45.75	4.01	236975	29.04	4.62	43171	31.41	2.38	73954	46.72	8.5	10870	85.54	22.7
HGF	100-120000	pg/mL	2452	13.41	12.19	12766	16.96	8.28	2154	20.58	1.7	785	7.96	11.57	283	10.19	20.14
MIF	100-120000	pg/mL	2230	19.69	7.89	11061	14.32	4.89	2209	12.31	1.68	574	8.59	13.68	513	63.56	62.9
sFas	100-150000	pg/mL	2486	27.52	6.36	12705	21.86	6.83	2779	26.23	1.37	3548	30.07	7.78	2217	51.45	22.99
sFasL	50-60000	pg/mL	1219	16.69	4.68	5898	20.4	2.92	1091	17.72	1.62	< MR	< MR	< MR	< MR	< MR	< MR
TRAIL	10-12000	pg/mL	252	13.53	4.72	1233	13.51	4.09	221	15.17	1.85	65	10.05	9.26	92	27.2	23.98
VEGF	50-60000	pg/mL	1127	25.4	4.27	6110	24.51	8.87	1115	18.99	3.76	< MR	< MR	< MR	< MR	< MR	< MR
IL-6	2-3000	pg/mL	49	14.49	9.34	226	22.21	17.12	53	18.81	4	16	12.3	14.64	< MR	< MR	< MR
IL-8	5-6000	pg/mL	122	8.85	5.21	605	11.32	3.73	110	9.31	1.98	33	9.31	8.37	6.71	25.35	40.54
TNFα	5-6000	pg/mL	114	21.44	5.52	562	16.31	6.81	110	16.78	2.28	13	19.54	8.14	6.41	26.96	40.75
TGFα	10-12000	pg/mL	237	16.15	6.26	1180	13.07	3.33	221	15.5	1.97	24	16.87	23.88	18	24.27	32.78
FGF2	50-50000	pg/mL	1101	11.17	8.98	5571	13.86	5.71	1066	12.75	3.89	55	11.36	68.81	79	19.8	40.04
SCF	20-30000	pg/mL	599	15.96	10.08	2813	13.38	4.75	550	14.69	1.93	58	11.5	10.22	50	24.27	22.5

Inter-assay imprecision results for all 24 tested markers based on FI as well as observed concentration (Obs Conc) results of assays 1-5 for synthetic quality controls QC 1, QC 2, standard 5 as well as for physiological serum pools 1 and 2. AFP: Alpha-fetoprotein; CA 125: Cancer antigen 125; CEA: Carcinoembryonic antigen; CV: Coefficient of variation; CYFRA 21-1: Cytokeratine 19-fragment; FGF2: Fibroblast growth factor-2; FI: Fluorescent intensity; HE4: Human epididymis protein 4; HGF: Hepatocyte growth factor; IL-6: Interleukin-6; MIF: Macrophage migration inhibitory factor; MR: Measuring range; Conc: Concentration; OPN: Osteopontin; QC 1: Quality control 1; SCF: Stem cell factor; sFas: Soluble Fas; sFasL: Soluble Fas-ligand; TGFα: Transforming growth factor-α; TNFα: Tumor necrosis factor-α; total PSA: Total prostate-specific antigen; TRAIL: Tumor necrosis factor related apoptosis-inducing ligand; VEGF: Vascular endothelial growth factor; β-HCG: β-human chorionic gonadotropin.

When samples were directly centrifuged after venous puncture and subsequently stored at 4 ℃ up to 48 h before measurements, biomarker levels showed relatively stable results. When storing the samples at room temperature for 6, 24 and 48 h before testing, stronger alterations were observed. Indeed, two, 0 and 16 biomarkers, respectively, failed the 70%-130% range of recovery. Detailed results are shown in Figures 3 and 4.

Testing in EDTA-plasma instead of serum samples did generally not affect the levels of biomarkers resulting in a median recovery of 110.8% [range: 60.7% (HGF)-272% (OPN)]. Here, four biomarkers failed the range of recovery (Figure 5).

DISCUSSION

Launching the multiplex magnetic bead assay into clinical routine and thus providing an insight of diverse corporal processes by means of biomarkers would greatly support

Table 4 Extended inter-assay imprecision

Biomarker	Measuring range	Unit	QC 1			QC 2			St 5			Pool 1			Pool 2		
			Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%
CEA	100-120000	pg/mL	2545	13.96	28.08	12727	20.57	25.67	2146	13.34	4.59	39391	6.94	7.38	560	14.32	25.77
AFP	500-600000	pg/mL	12038	23.54	20.99	56960	19.11	10.29	10754	21.43	3.25	493993	12.38	18.42	1489	33.13	63.64
Total PSA	50-60000	pg/mL	1421	34.29	31.87	6172	17.44	13.78	1052	18.72	3.67	8246	16.03	12.46	68	130.09	151.66
CA 15-3	0.5-600	U/mL	12	30.11	18.58	62	29.25	12.98	11	34.01	2.93	33	23.72	17.26	26	30.49	27.59
CA 19-9	2-3000	U/mL	64	22.71	17.91	329	11.98	19.1	54	19.2	2.71	36	15.18	26.88	10	16.18	36.06
CA 125	2-4000	U/mL	74	14.42	22.04	370	12.5	11.07	68	17.29	2.42	317	7.72	12.62	6.13	18.13	37.03
β-HCG	0.2-400	mU/mL	7.85	20.62	20.74	42	16.95	16.23	7	26.88	2.69	1.2	29.09	52.2	0.55	17.3	74.36
CYFRA 21-1	500-900000	pg/mL	24985	69.07	36.07	152731	81.41	52.97	16601	53.61	2.5	8627	50.45	55.77	3506	31.82	30.8
HE4	2000-3000000	pg/mL	55766	22.11	21.46	295050	19.92	10.63	55043	22.45	2.1	2288	19.7	68.01	2590	22.73	54.77
Prolactin	500-600000	pg/mL	11952	23.76	21.49	60823	13.52	9.33	10863	14.81	2.05	12191	17.16	20.41	10602	29.43	28.46
Leptin	500-600000	pg/mL	13001	29.19	15.47	64533	19.61	8.82	11020	23.3	1.88	14622	16.75	18.61	9145	31.06	27.03
OPN	2000-3000000	pg/mL	57576	58.11	28.1	240688	24.08	13.88	42845	25.18	5.71	75488	41.77	25.01	10700	62.83	47.91
HGF	100-120000	pg/mL	2546	15.19	20.7	13471	19.05	12.85	2171	22.65	2.04	894	7.18	27.83	436	29.6	49.81
MIF	100-120000	pg/mL	2263	46.52	45.1	12332	20.48	18.05	2220	23.01	2.93	876	62.88	48.48	517	45.1	47.35
sFas	100-150000	pg/mL	2533	27.15	18.62	13212	20.06	12.63	2775	27.38	1.77	4005	29.39	21.39	2472	39.44	28.26
sFasL	50-60000	pg/mL	1245	19.09	20.91	6023	21.37	6.63	1093	22.37	1.98	< MR	< MR	< MR	< MR	< MR	< MR
TRAIL	10-12000	pg/mL	253	20.17	21.78	1269	17.13	10.13	220	21.55	2.05	72	11.04	32.6	98	26.7	35.57
VEGF	50-60000	pg/mL	1146	46.21	23	6155	45.49	8.97	1100	43.93	3.26	< MR	< MR	< MR	< MR	< MR	< MR
IL-6	2-3000	pg/mL	54	17.46	26.94	268	17.39	23.92	54	24.51	5.54	21	14.19	41.05	< MR	< MR	< MR
IL-8	5-6000	pg/mL	124	75.72	21.22	668	45.79	22.2	111	30.7	3.13	26	36.91	38.62	6.70	90.89	40.54
TNFα	5-6000	pg/mL	117	18.6	23.55	583	16.6	11.16	111	19.41	2.52	16	15.5	28.1	7.85	22.35	49.24
TGFα	10-12000	pg/mL	238	18.16	21.43	1210	19.49	7.74	221	22.29	2.56	27	17.64	45.12	18	19.22	48.85
FGF2	50-50000	pg/mL	1104	25.12	23.52	5708	17.67	9.9	1068	19.05	3.55	68	9.43	49.91	80	15.21	32.41
SCF	20-30000	pg/mL	603	12.44	19.52	2951	16.66	11.26	549	17.88	2.01	79	31.9	40.75	59	22.35	32.17

Inter-assay imprecision results for all 24 tested markers based on FI as well as observed concentration (Obs Conc) results of all assays 1-10 for synthetic quality controls QC 1, QC 2, standard 5 as well as for physiological serum pools 1 and 2. AFP: Alpha-fetoprotein; CA 125: Cancer antigen 125; CEA: Carcinoembryonic antigen; CV: Coefficient of variation; CYFRA 21-1: Cytokeratine 19-fragment; FGF2: Fibroblast growth factor-2; FI: Fluorescent intensity; HE4: Human epididymis protein 4; HGF: Hepatocyte growth factor; IL-6: Interleukin-6; MIF: Macrophage migration inhibitory factor; MR: Measuring range; Conc: Concentration; OPN: Osteopontin; QC 1: Quality control 1; SCF: Stem cell factor; sFas: Soluble Fas; sFasL: Soluble Fas-ligand; TGFα: Transforming growth factor-α; TNFα: Tumor necrosis factor-α; total PSA: Total prostate-specific antigen; TRAIL: Tumor necrosis factor related apoptosis-inducing ligand; VEGF: Vascular endothelial growth factor; β-HCG: β-human chorionic gonadotropin.

tumor diagnostics and differential diagnosis[20,21]. Here, an appropriate pre-analytical evaluation is indispensible in order to obtain reliable clinical results in the future[2,3].

As well-known for many research-use-only (RUO)-assays, there often is only limited data available concerning the methodological performance despite being distributed and used for study and research purposes[20-22]. This is quite relevant for the scientific community as the same names of the parameters are used in these compound tests as in regular *in-vitro* diagnostic (IVD) labeled assays which are commonly used in clinical laboratory routine diagnostics for which the methodical quality and clinical validity has been investigated thoroughly in most cases.

Certainly, the assay of the present study has to be perceived as a complex method consisting of as many different tests as markers are included. It seems to be quite challenging for manufacturers to optimize all markers in a multiparametric platform and to avoid interactions between them. This is all the more obvious by the conspicuous variety of the measured biomarkers regarding their chemical and physical characteristics. Thus, to evaluate the measurement quality of this assay the single markers have to be interpreted independently.

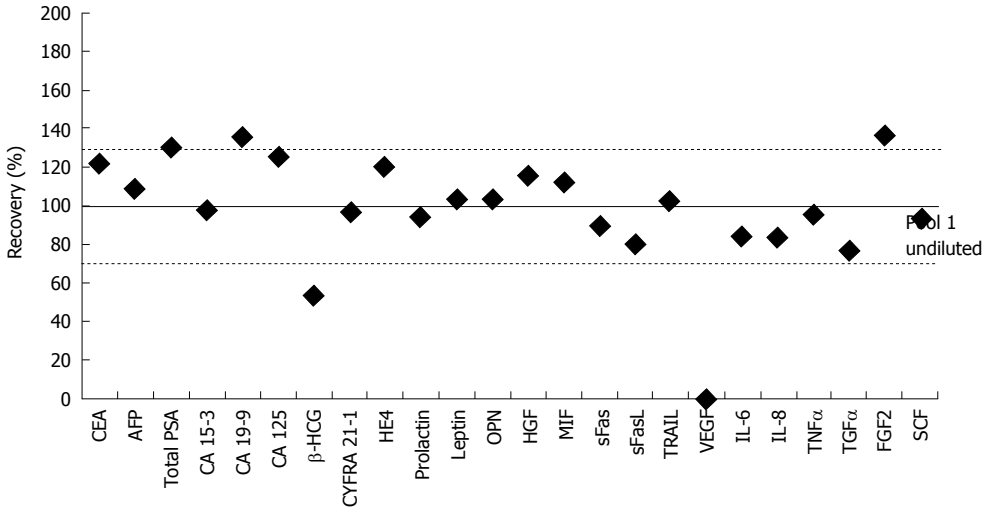

Figure 1 Dilution recovery. Recoveries of a 50% dilution of serum pool 1 for all 24 biomarkers. The values are observed concentration-based and the corresponding measured undiluted pool 1 values were taken as reference. Horizontal lines depict the acceptable range of recovery. The dilution of VEGF fell below the measuring range. AFP: Alpha-fetoprotein; CA 125: Cancer antigen 125; CEA: Carcinoembryonic antigen; CV: Coefficient of variation; CYFRA 21-1: Cytokeratine 19-fragment; FGF2: Fibroblast growth factor-2; FI: Fluorescent intensity; HE4: Human epididymis protein 4; HGF: Hepatocyte growth factor; IL-6: Interleukin-6; MIF: Macrophage migration inhibitory factor; MR: Measuring range; Conc: Concentration; OPN: Osteopontin; QC 1: Quality control 1; SCF: Stem cell factor; sFas: Soluble Fas; sFasL: Soluble Fas-ligand; TGFα: Transforming growth factor-α; TNFα: Tumor necrosis factor-α; total PSA: Total prostate-specific antigen; TRAIL: Tumor necrosis factor related apoptosis-inducing ligand; VEGF: Vascular endothelial growth factor; β-HCG: β-human chorionic gonadotropin.

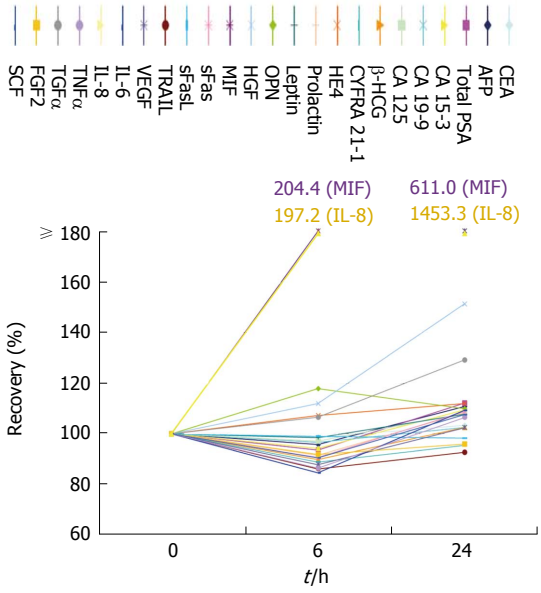

Figure 2 Preanalytical factors: Storage before centrifugation. Behavior of biomarkers undergoing different pre-analytical conditions: Blood samples were stored at room temperature for 0, 6 and 24 h before centrifugation, definitive storage at -80 ℃ and measurement. Values are FI-based and depicted as recoveries corresponding to the values of directly centrifuged and frozen serum as reference. AFP: Alpha-fetoprotein; CA 125: Cancer antigen 125; CEA: Carcinoembryonic antigen; CV: Coefficient of variation; CYFRA 21-1: Cytokeratine 19-fragment; FGF2: Fibroblast growth factor-2; FI: Fluorescent intensity; HE4: Human epididymis protein 4; HGF: Hepatocyte growth factor; IL-6: Interleukin-6; MIF: Macrophage migration inhibitory factor; MR: Measuring range; Conc: Concentration; OPN: Osteopontin; QC 1: Quality control 1; SCF: Stem cell factor; sFas: Soluble Fas; sFasL: Soluble Fas-ligand; TGFα: Transforming growth factor-α; TNFα: Tumor necrosis factor-α; total PSA: Total prostate-specific antigen; TRAIL: Tumor necrosis factor related apoptosis-inducing ligand; VEGF: Vascular endothelial growth factor; β-HCG: β-human chorionic gonadotropin.

All in all, ten plates were run. The first five kits were ordered in one batch and applied subsequently under strictly identical conditions. The following plates were ordered and run six months later under the same standards of procedure. However, we perceived a striking discrepancy when comparing the results of the first five and the last five plates. As the lot number was the same, we assume an influence of surrounding conditions, *e.g.*, room temperature, pipettes used and different laboratory staff constellation. In order to represent accurate and relevant results in our main method evaluation, we divided our evaluation into two steps: First, we considered only the first five assays as they were done under homogeneous conditions. In the second step, an overall analysis of all assays was performed. Nevertheless, this critical fact elucidates once more the importance of standardized procedures in clinical routine laboratories, the application of internal quality controls and the participation in external quality assessment programs[3,18].

In general, the assay showed an acceptable intra- and inter-assay imprecision. Apart from the synthetic internal controls QC 1 and QC 2 we included the physiological external control samples pool 1 and pool 2 as a further reference source. In comparison to the internal controls QC 1 and QC 2 this resulted in an interesting finding. As expected, the method precision was slightly more accurate for the synthetic samples. Although evidently the composition of serum pools is more complex, this physiological type of quality control is more relevant as it reflects the situation of the clinical samples more accurately. Nevertheless, the comparability between synthetic and physiological samples was still given. However, we perceived higher variation in samples with lower con-

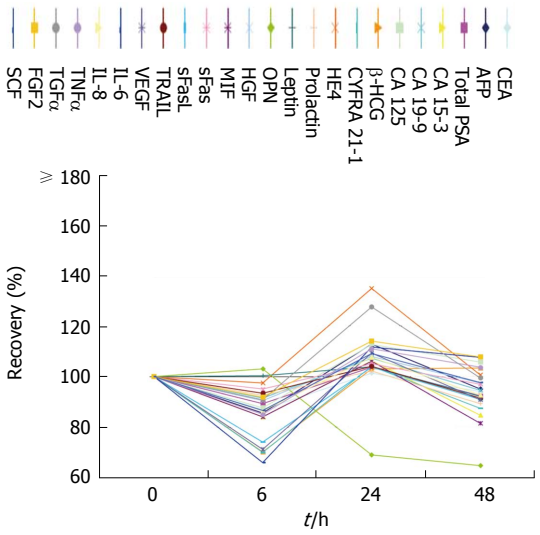

Figure 3 Preanalytical factors: Storage after centrifugation at 4 ℃. Behavior of biomarkers undergoing different pre-analytical conditions: Blood samples were stored for 0, 6, 24 and 48 h after centrifugation at 4 ℃ before definitive storage at -80 ℃ and measurement. Values are FI-based and depicted as recoveries corresponding to the values of directly centrifuged and frozen serum as reference. AFP: Alpha-fetoprotein; CA 125: Cancer antigen 125; CEA: Carcinoembryonic antigen; CV: Coefficient of variation; CYFRA 21-1: Cytokeratine 19-fragment; FGF2: Fibroblast growth factor-2; FI: Fluorescent intensity; HE4: Human epididymis protein 4; HGF: Hepatocyte growth factor; IL-6: Interleukin-6; MIF: Macrophage migration inhibitory factor; MR: Measuring range; Conc: Concentration; OPN: Osteopontin; QC 1: Quality control 1; SCF: Stem cell factor; sFas: Soluble Fas; sFasL: Soluble Fas-ligand; TGFα: Transforming growth factor-α; TNFα: Tumor necrosis factor-α; total PSA: Total prostate-specific antigen; TRAIL: Tumor necrosis factor related apoptosis-inducing ligand; VEGF: Vascular endothelial growth factor; β-HCG: β-human chorionic gonadotropin.

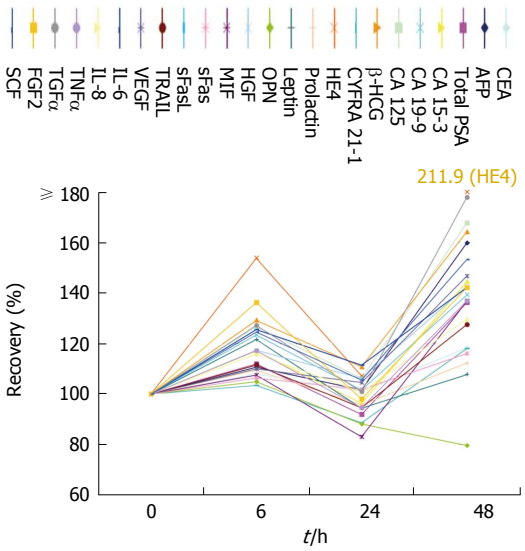

Figure 4 Preanalytical factors: Storage after centrifugation at room temperature. Behavior of biomarkers undergoing different pre-analytical conditions: Blood samples were stored for 0, 6, 24 and 48 h after centrifugation at room temperature before definitive storage at -80 ℃ and measurement. Values are FI-based and depicted as recoveries corresponding to the values of directly centrifuged and frozen serum as reference. AFP: Alpha-fetoprotein; CA 125: Cancer antigen 125; CEA: Carcinoembryonic antigen; CV: Coefficient of variation; CYFRA 21-1: Cytokeratine 19-fragment; FGF2: Fibroblast growth factor-2; FI: Fluorescent intensity; HE4: Human epididymis protein 4; HGF: Hepatocyte growth factor; IL-6: Interleukin-6; MIF: Macrophage migration inhibitory factor; MR: Measuring range; Conc: Concentration; OPN: Osteopontin; QC 1: Quality control 1; SCF: Stem cell factor; sFas: Soluble Fas; sFasL: Soluble Fas-ligand; TGFα: Transforming growth factor-α; TNFα: Tumor necrosis factor-α; total PSA: Total prostate-specific antigen; TRAIL: Tumor necrosis factor related apoptosis-inducing ligand; VEGF: Vascular endothelial growth factor; β-HCG: β-human chorionic gonadotropin.

centrations of the biomarkers, particularly seen in the imprecision results of pool 2 where most marker concentrations were below or at the lower end of the accepted range.

Comparing the precision data provided by the manufacturer (range: 4.9% to 15.0% and 4.1% to 16.2% for intra- and inter-assay imprecision, respectively) with our results a very good accordance concerning the intra-assay results was observed.

The inter-assay imprecision is partly higher in our tests. However, for most markers we could achieve the corresponding coefficients of variation considering the results of the quality controls provided by the manufacturer. CYFRA 21-1, a highly valuable tumor marker for non small cell lung cancer[16,23,24], was found to be the only marker exceeding the 20% limit of CV concurring with the given imprecision by the manufacturer where it also yields the maximum coefficient of variation in the panel. As the marker concentration is within the appropriate range and the variation is found to be not acceptable we assume a non-applicability of this biomarker in the assessed method. In our higher concentrated pool 1, five markers were found to be measured with a non-acceptable CV. Close observation of these biomarkers revealed that the observed concentrations were very low, even below the first standard (such as for FGF2) indicating only limited clinical relevance in these cases. In conclusion, with an inter-assay imprecision in a reasonable range between 4.59% (total PSA)-23.88% (TGFα), this is a rather satisfying result.

Comparing the values of variance of fluorescence intensity and observed concentration revealed some striking discrepancies. This phenomenon is obviously based on the transformation of measured fluorescence intensity into biomarker concentration, which is neither linear nor predetermined to be equal in all the tests, but corresponding to the course of the standard curve calculated anew for every single test. FI values are matched to the concentrations that are provided by the standard curve. Thus, the same FI values in two different plates could lead to two different concentrations and are therefore less comparable when it comes to a crossover comparison between the plates. This phenomenon is shown in the attached Table 3 comparing FI values and observed concentration-based CVs. Hence, the inter-assay imprecision based on concentration values provided a more relevant result for the methodological evaluation.

Testing the dilution linearity, our study yields satisfying results with a tendency to a recovery in the upper field of the defined acceptable range.

Within the Bio-Plex® 200 System, it is possible to vary the minimal number of events needed for measurement. With 50 events as minimum per bead we chose a

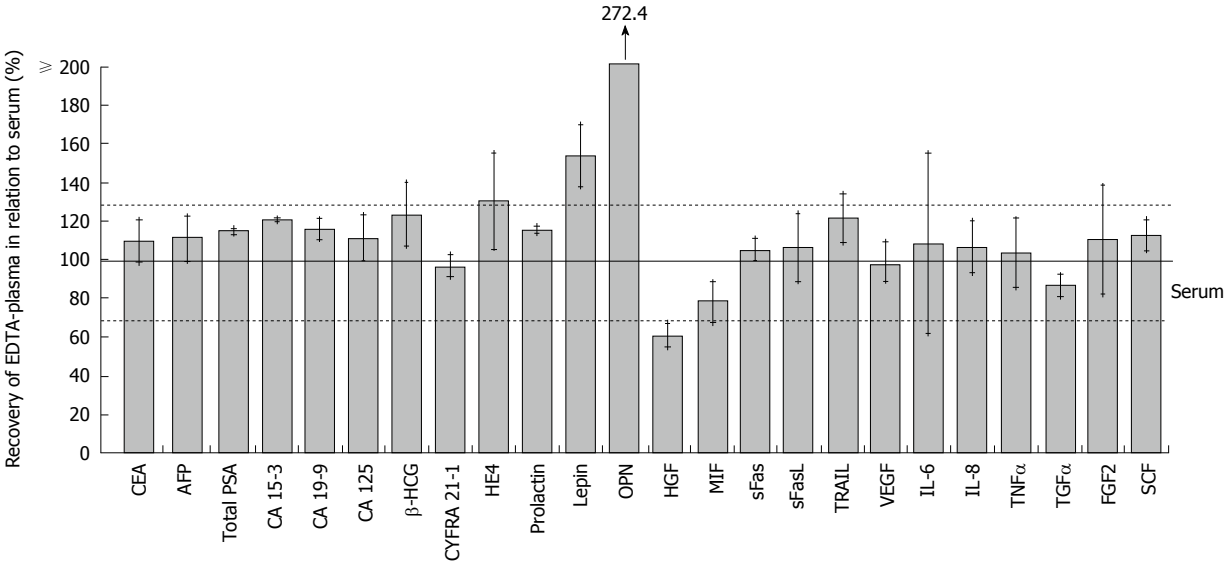

Figure 5 Comparison between serum and EDTA-plasma. Measured serum levels are defined as 100% recovery. The acceptable range is indicated by the horizontal lines and standard deviation of the recoveries is given for each marker. AFP: Alpha-fetoprotein; CA 125: Cancer antigen 125; CEA: Carcinoembryonic antigen; CV: Coefficient of variation; CYFRA 21-1: Cytokeratine 19-fragment; FGF2: Fibroblast growth factor-2; FI: Fluorescent intensity; HE4: Human epididymis protein 4; HGF: Hepatocyte growth factor; IL-6: Interleukin-6; MIF: Macrophage migration inhibitory factor; MR: Measuring range; Conc: Concentration; OPN: Osteopontin; QC 1: Quality control 1; SCF: Stem cell factor; sFas: Soluble Fas; sFasL: Soluble Fas-ligand; TGFα: Transforming growth factor-α; TNFα: Tumor necrosis factor-α; total PSA: Total prostate-specific antigen; TRAIL: Tumor necrosis factor related apoptosis-inducing ligand; VEGF: Vascular endothelial growth factor; β-HCG: β-human chorionic gonadotropin.

compromise between a time-effective measurements and sufficiently precise results. Obviously, the accuracy of measurements increases with higher number of events. Nevertheless, our findings could achieve acceptable CV values in most cases despite the low minimum number of events set. Therefore, we did not compare absolute number of detected events and their calculated CVs.

The MILLIPLEX® MAP Human Circulating Cancer Biomarker Magnetic Bead Panel 1 offers a wide spectrum of applicable biomarkers. However, it is obviously neither clinically relevant nor cost-effective to apply the complete panel in diagnostics. After definition of the relevant markers for each tumor type, the panel must be focused and applied as a biomarker pattern of clinical interest depending on the contemplated entity of disease.

For implementation into clinical diagnostics, further studies evaluating its performance in a large cohort of cancer patients and appropriate control groups, which are relevant for differential diagnosis, *i.e.*, healthy individuals and patients with organ related benign disease, are definitely required. Currently, we are performing such clinical validation studies with cohorts of patients suffering from gastrointestinal, gynecological and urological cancers.

Apart from the method quality itself, preanalytical handling of samples prior to analysis in the laboratory can influence the final results, as is known for several research and routine parameters[25-27]. Hence, we examined the stability of the tested markers. As often observed in the clinical routine, samples are not directly transferred to the central laboratory and instead remain exposed to room temperature without centrifugation. In order to depict this highly relevant situation, samples were centrifuged after 6 and 24 h. However, most markers remained stable, except MIF and IL-8. These two markers presented a considerable increase in marker levels. Our findings for IL-8 agree with the recommendations made by Hoch *et al*[28] to centrifuge the blood samples within less than 2 h to avoid interactions between IL-8 and blood cells. An increase of MIF levels in samples, which were not directly prepared, is also predescribed by Sobierajski *et al*[29]. These pre-analytical facts must be observed by the clinicians as prolonged storage before processing could lead to fatal misinterpretation in these markers.

Sample storage up to 48 h after centrifugation at a temperature of 4 ℃ showed a good outcome for all markers except OPN, which presented a decline after 6 h storage.

Storage at 25 ℃ after centrifugation showed a stronger effect on marker levels. While stability is given until 24 h, a more or less increase of marker concentrations can be observed after longer storage time. For example, the recovery of HE4 rises over 200% after 48 h. Again, OPN is the only marker showing a decline to nearly 80% in recovery supporting our above mentioned findings. Also, Cristaudo *et al*[30] found an instable performance of OPN after storage of serum samples at room temperature.

Furthermore, we observed good comparability between serum and EDTA-plasma samples. Only OPN and leptin-recoveries exceeded the 130% mark of the accepted range. These results correspond with the findings of Lanteri *et al*[31] and Gröschl *et al*[32] where an increase of these biomarkers in plasma samples compared with serum samples was observed. HGF presented a recovery of 60.7% as the unique marker undergoing the accepted 70% mark, also concurring with previous HGF stability analysis[33].

In our study, the evaluated method is demonstrated

to be a stable and precise tool for detection of most biomarkers included in the kit. Unfortunately, for CYFRA 21-1, the method did not achieve acceptable inter-assay precision values. This should be further investigated. In general, we recommend that "research use only"-tests are assessed before implementation into further research and clinical routine. Here, certain preconditions, such as ordering tests in a batch, use of physiological quality controls in addition to the provided control samples as well as relevant pre-analytical aspects of some markers, should be observed.

All in all, this study shows that the MILLIPLEX® MAP Human Circulating Cancer Biomarker Magnetic Bead Panel 1 could offer new diagnostic perspectives while further studies are necessary to show its clinical applicability, usefulness and comparability with established routine assays.

ACKNOWLEDGMENTS

We are grateful for access to the Biorad and Luminex instruments of the laboratory of Professor Knolle and the laboratory of Professor Hornung at the University Hospital Bonn, where the analyses were performed. This work is part of the doctoral theses of Hermann N and Dreßen K.

COMMENTS

Background

Cancer is a global health problem resulting in about eight million deaths each year. Tumor markers as additional diagnostic tools, which are easily assessable in blood, have been launched in the last century and with them a large variety of detecting methods. Of these, enzyme-linked immunosorbent assays are the standard methods in clinical routine today, while multiplexing has become interesting for cancer research as it is a quick, cheap, less-volume-wasting, easy-to-handle but still precise tool for parallel measurement of multiple markers. This goes hand in hand with the fact that nowadays cancer is perceived as a complex disease involving multiple processes. The authors investigated the MILLIPLEX® MAP Human Circulating Cancer Biomarker Magnetic Bead Panel 1, purchased from Millipore, which was specially designed for cancer diagnostics, on its methodical performance.

Research frontiers

Most striking is the fact that many different assays are used for research purpose, although they are not that strictly proven as established methods for *in-vitro* diagnostics. However, research results are assigned to a clinical setting. Therefore, a thorough methodical investigation using physiological samples has to be performed.

Innovations and breakthroughs

Previous investigations of the multiplex technology used by Millipore showed good correlations to single ELISAs of each tested biomarker. However, here only a few markers were tested in parallel. Furthermore, this specially designed kit with the possibility to measure 24 biomarkers from the areas of angiogenesis, immunology, apoptosis as well as established and auspicious tumor markers has not been tested on its entirety regarding methodological performance and stability. In the investigations, the authors used serum pools as physiological control samples and most markers showed good results.

Applications

This study allows other users to assess the quality and applicability of the assay and to conduct further clinical studies on methodically solid bedrock.

Terminology

All substances that can be measured in cancer patients and which reveal a malignant disease or contribute to its prognosis or treatment are called tumor markers. Multiplexing is one of the detection methods for tumor markers which are assessed in body fluids and it allows the parallel measurement of multiple markers. An ELISA is an enzyme-linked immunosorbent assay which is another detection method for tumor markers but limited to the measurement of only one marker per test. Both are antibody-based detection procedures and enzymatic color-reactions are used to quantify the results.

Peer review

The manuscript is very well presented and highlights factors influencing the measurements of the key performance indicators of the multiplex cancer biomarker panel, which constitutes a highly interesting study. The findings from the comparison of the critical measurement parameters between physiological sera in parallel with synthetic internal controls will be of particular interest to a wide audience. The technical details are clearly defined and the interpretations are thorough with robust scientific conclusions.

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