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EDITORIAL

New developments and controversies in iron metabolism and iron chelation therapy

Christina N Kontoghiorghe, George J Kontoghiorghes

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Author contributions: Kontoghiorghe CN contributed to the literature background on recent developments on iron metabolism and chelation and critically reviewed the clinical and other aspects of the manuscript; Kontoghiorghes GJ designed, wrote and edited the manuscript including all aspects on controversies, the mechanisms of iron chelation therapy and also iron metabolism and toxicity.

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Abstract

Iron is essential for all organisms including microbial, cancer and human cells. More than a quarter of the human population is affected by abnormalities of iron metabolism, mainly from iron deficiency and iron overload. Iron also plays an important role in free radical pathology and oxidative damage which is observed in almost all major diseases, cancer and ageing. New developments include the complete treatment of iron overload and reduction of morbidity and mortality in thalassaemia using deferiprone and selected deferiprone/deferoxamine combinations and also the use of the maltol iron complex in the treatment of iron deficiency anaemia. There is also a prospect of using deferiprone as a universal antioxidant in non iron overloaded diseases such as neurodegenerative, cardiovascular, renal, infectious diseases and cancer. New regulatory molecules of iron metabolism such as endogenous and dietary chelating molecules, hepcidin, mitochondrial ferritin and their role in health and disease is under evaluation. Similarly, new mechanisms of iron deposition, removal, distribution and toxicity have been identified using new techniques such as magnetic resonance imaging increasing our understanding of iron metabolic processes and the targeted treatment of related diseases. The uniform distribution of iron in iron overload between organs and within each organ is no longer valid. Several other controversies such as the toxicity impact of non transferrin bound iron *vs* injected iron, the excess levels of iron in tissues causing toxicity and the role of chelation on iron absorption need further investigation. Commercial interests of pharmaceutical companies and connections to leading journals are playing a crucial role in shaping worldwide medical opinion on drug sales and use but also patients' therapeutic outcome and safety. Major controversies include the selection criteria and risk/benefit assessment in the use of deferasirox in thalassaemia and more so in idiopathic haemochromatosis, thalassaemia intermedia and ex-thalassaemia transplanted patients who are safely treated with venesection. Iron chelating drugs can override normal regulatory pathways, correct iron imbalance and minimise iron toxicity. The use of iron chelating drugs as main, alternative or adjuvant therapy

is in progress in many conditions, especially those with non established or effective therapies.

Key words: Iron metabolism; Iron chelation therapy; Deferiprone; Deferoxamine; Deferasirox; Iron diseases; Medical journals; Controversies

Core tip: Abnormalities of iron metabolism including iron deficiency and overload affect more than a quarter of the world's population. Iron also plays a major role in free radical pathology and associated tissue damage. Iron chelating drugs can override normal regulatory pathways, correct iron imbalance and minimise iron toxicity. Deferiprone and especially its combination with deferoxamine can completely treat iron overload in thalassaemia. Deferiprone can minimise the toxic effects of pathological iron in neurodegenerative, renal and other diseases. Controversies in the risk/benefit assessment for the use of deferasirox in many conditions appear to involve commercial influence on academic journals and physicians.

Kontoghiorghe CN, Kontoghiorghes GJ. New developments and controversies in iron metabolism and iron chelation therapy. *World J Methodol* 2016; 6(1): 1-19 Available from: URL: http://www.wjgnet.com/2222-0682/full/v6/i1/1.htm DOI: http://dx.doi.org/10.5662/wjm.v6.i1.1

INTRODUCTION

Iron is an essential metal found in all living organisms including microbial, cancer and normal human cells. More than a quarter of the human population is affected at some stages in their life by iron deficiency. Similarly, many millions suffer from other abnormalities of iron metabolism, such as iron overload in hereditary haemochromatosis which is caused by increased iron absorption and iron overload in thalassaemia which is a result of chronic transfusions[1,2]. Iron also plays an important catalytic role in free radical pathology and oxidative damage which is observed in almost all major iron loaded and non iron loaded diseases such as cardiovascular, neurodegenerative, hepatic and renal diseases, as well as in cancer and ageing[3].

Most of the diseases related to iron metabolic imbalance can be treated using established and effective therapeutic approaches, *e.g.,* iron supplementation for the treatment of iron deficiency anaemia and venesection in hereditary haemochromatosis. Iron overload in thalassaemia is more difficult to treat using chelation therapy and the same applies for the treatment of the anaemia of chronic disease in many conditions such as cancer, rheumatoid arthritis and haemodialysis, where oral or intravenous iron, with or without erythropoietin combination may be used.

Most of the therapies of abnormal iron metabolism described above are widely applied in developed countries but there are financial constrains for their use by patients in the developing countries. In particular the treatment of thalassaemia using regular transfusions and chelation therapy and also the use of erythropoietin in the anaemia of chronic disease is not affordable for the vast majority of patients in the developing countries[4,5].

The disease with the highest mortality and morbidity rate related to iron metabolic disorders worldwide is thalassaemia, which is found mainly in developing countries of South East Asia, Middle East and Mediterranean. More than 100000 thalassaemia babies are born every year with 9000 in India alone, most dying without treatment[4-6]. Despite that health facilities including blood transfusions are improving in developing countries, the cost of the chelating drugs is still not affordable for most patients living in these countries and therefore life expectancy is low[5]. Usually, non-transfused thalassaemia patients die by the age of 7 years and transfused but not chelated thalassaemia patients die by the age of 20 years, mainly from congestive cardiac failure due to cardiac iron overload toxicity[7,8]. The life expectancy of thalassaemia patients receiving chelation therapy increases substantially and many patients adhering to the chelation protocol with deferiprone (L1) and deferoxamine (DF) are now exceeding the age of 50 years (Figure 1)[9].

However, despite the wide availability of the chelating drugs DF, L1 and deferasirox (DFRA) in developed countries and indications that the use of appropriate effective protocols can lead to the complete treatment of iron overload, their application to thalassaemia patients appears to be influenced by physician decisions associated with literature rivalry and commercial interests[5]. As a result of the commercial interference and influence which is mainly caused by the manufacturers of chelating drugs and their marketing methods the overall treatment outcome, safety and survival of the thalassaemia patients is greatly affected[5].

Clinical trials and preclinical studies suggest that there are increasing prospects of using chelation and in particular L1 as a universal antioxidant in non iron overload diseases such as neurodegenerative, cardiovascular, renal and infectious diseases as well as cancer and ageing[10,11].

The discovery of new regulatory molecules of iron metabolism such as endogenous and dietary chelating molecules as well as the proteins hepcidin, ferroportin, mitochondrial ferritin and their role in normal and iron overload disease states is subject to continuous investigation[1,2]. Similarly, the identification of new mechanisms of iron deposition, removal, distribution and toxicity have increased further our understanding of iron metabolic processes and improved the use of specific targeting treatments in iron metabolic diseases.

In general, the acquisition and distribution of important and significant knowledge for all diseases is becoming a major issue in the treatment outcome and

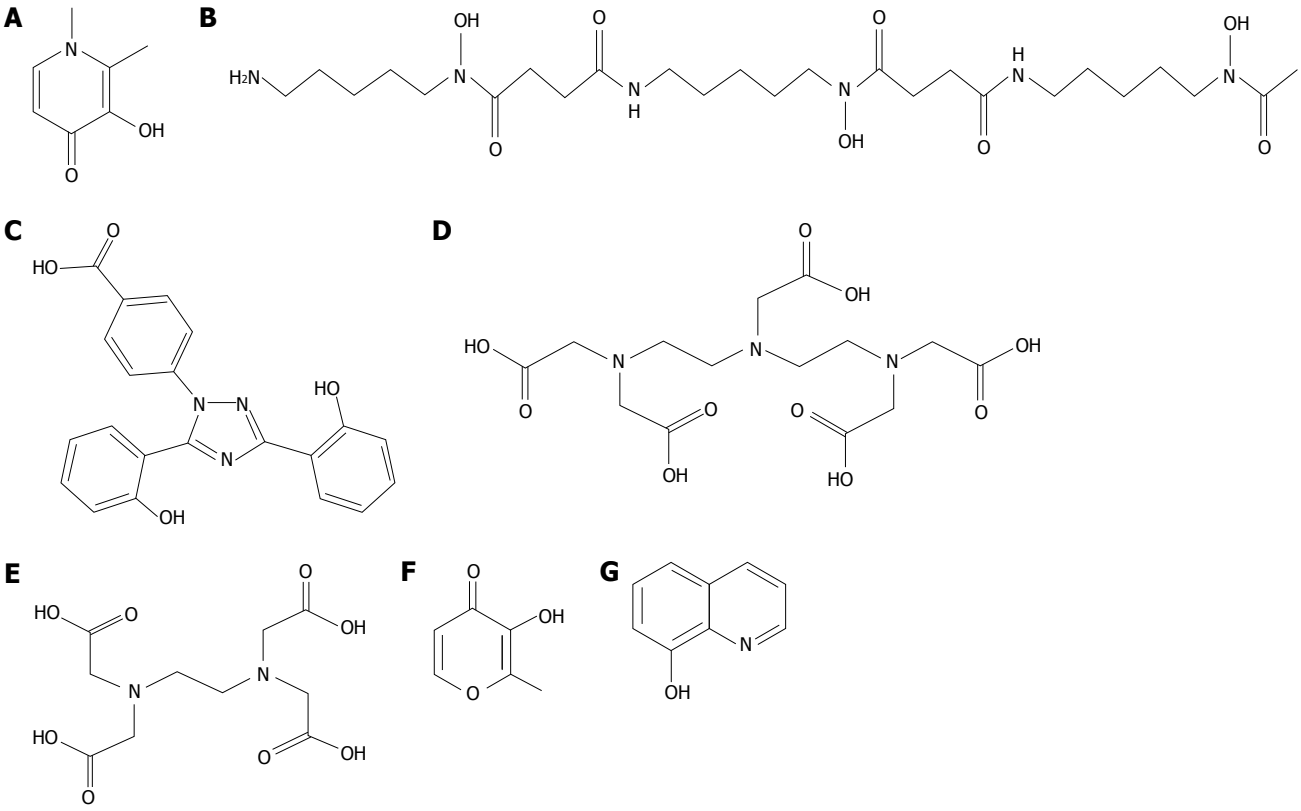

Figure 1 The chemical structure of the iron chelating drugs. L1 (A), DF (B) and DFRA (C) are currently used for the treatment of thalassaemia and other transfusional iron loading conditions. DTPA (D) and EDTA (E) have been previously used for the treatment of iron overload but are now used for the detoxification of toxic metals and in particular EDTA in alternative medicine. The maltol (F) iron complex is used for increasing iron absorption and 8-hydroxyquinoline (G) is a lipophilic chelator used for radiolabeling in diagnostic medicine and for experimental purposes. L1: Deferiprone; DF: Deferoxamine; DFRA: Deferasirox; DTPA: Diethylenetriaminepentaacetic acid; EDTA: Ethylenediaminetetraacetic acid.

safety of patients. Within this context many controversies related to the diagnosis and treatment of iron abnormalities have been identified involving influence by commercial interests, especially by pharmaceutical companies and related support by a section of leading medical journals in the selection and promotion of drug treatments with high risk and low benefit outcomes for patients[5].

The controversies are also extended to research findings in relation to basic mechanisms of chelating drug action and also to iron metabolism and toxicity. These issues include for example the impact of toxicity of non transferrin bound iron (NTBI) found mainly in iron loaded patients *vs* the lack of toxicity of injected iron used in anaemic patients. Similarly, the role and limitations of the function of hepcidin as a universal regulator of normal and abnormal iron metabolism are also questioned.

Controversies associated to basic mechanisms of drug toxicity especially in the case of DFRA, for example in relation to the increased absorption of toxic metals such as Al are still unanswered[12,13]. Furthermore, the promotion of the use of DFRA instead of the safer and more effective use of venesection is also questioned especially in relation to the treatment of idiopathic haemochromatosis, thalassaemia intermedia and in ex-thalassaemia transplanted patients. Another issue in relation to the use of DFRA which are also controversial is its effect in the mortality and morbitidy rate of transfused patients with myelodysplastic, myelofibrosis, sickle cell anaemia and also in non iron loaded conditions.

It appears that in general many controversial issues including the risk/benefit assessment of the use of iron chelating drugs worldwide in different conditions seem to be based on the marketing policies and commercial influence of pharmaceutical companies and not the therapeutic needs and safety of patients[5]. Within this context some recent developments in iron metabolism will be reviewed with emphasis on the topics and issues that affect the treatment of patients with iron metabolic disorders both in developed and developing countries. Similarly, recent developments that affect the treatment outcome and safety of patients including commercial influence and other non medical factors will also be discussed.

MOLECULAR ASPECTS OF IRON METABOLIC DISORDERS

The molecular aspects of iron including its chemical and biochemical properties are important in the understanding of iron metabolism and chelation therapy. Iron is generally found in the ferrous Fe (Ⅱ) and ferric Fe (Ⅲ) oxidation states in the human body. For example, it is transported in the plasma by transferrin and stored in ferritin and haemosiderin in the ferric form, whereas it is

found in the ferrous form in haemoglobin and myoglobin bound to oxygen and also in other proteins involved in redox reactions[14]. It can also sometimes be found in the Fe (Ⅳ) form in haem, which is associated with pathological effects[15].

The solubility of iron under physiological conditions is an important property for its metabolic functions and toxicity. Ferric iron hydrolyses at pH 7.4 forming insoluble oxohydroxy polynuclear complexes which precipitate. The solubility of ferric iron is extremely low and at physiological pH 7.4 is estimated to be less than 10^{-18} mol/L. The amount of soluble iron is negligible compared to the iron turnover needed for the different physiological functions and in particular for the production of haemoglobin. The solubility of iron (Ⅲ) can increase by different methods such as by decreasing the pH, reducing iron (Ⅲ) to iron (Ⅱ) or using chelating agents. Examples of such processes in physiological conditions is the solubilisation of iron in food in the acidic medium of the stomach, reduction of iron (Ⅲ) to iron (Ⅱ) in the duodenum by a cytochrome b-like ferrireductase (Dcytb) and chelation and transport of iron (Ⅲ) by transferrin[1,2,14]. Another method for the solubility of polynuclear iron is achieved intracellularly by ferritin, which encloses the insoluble oxohydroxy polynuclear iron within a soluble protein shell[1,2,16,17].

The redox changes of iron are of biological and toxicological importance. In particular, iron toxicity arises mainly from the catalytic activity of ferrous iron in the formation of free radicals and other reactive oxygen species which have been shown to cause molecular damage to all organic biomolecules including lipids, sugars, proteins and DNA[3,18]. Such biomolecular damage can lead to subcellular, cellular, tissue and organ damage, which can be permanent or reversible[19]. Ferric iron cannot catalyse the production of free radicals and is mostly not toxic unless it is reduced. However, *in vivo* iron (Ⅲ) can be reduced to iron (Ⅱ) by reducing agents such as ascorbic acid and other organic acids and consequently catalyse free radical production[20].

The presence of excess iron is considered a potential source of toxicity which can be expressed at the molecular, subcellular, cellular, tissue and organ level. Such forms of excess iron in polynuclear form include ferritin, haemosiderin and NTBI. Usually the damage to tissues and organs in iron loaded diseases depends on the concentration of excess stored iron mainly in the form of haemosiderin. At low iron concentrations of excess iron such damage is considered reversible due to the effective antioxidant protection mechanisms and antioxidant molecules and also the efficiency of the repair mechanisms[19]. However, at high concentrations excess iron can cause permanent damage and can be fatal, *e.g.*, in cardiac iron overload in thalassaemia[7,8].

Under normal conditions iron is essential to all cells and plays an important role in physiological functions including the growth and development of the body. It is absorbed from ingested food in small quantities of about 1-2 mg/d. The total body iron of normal adult humans is estimated at 3-5 g. Most of the iron is found in blood in the form of haemoglobin (58%) in red blood cells, as myoglobin (9%) in muscle tissue and as intracellular ferritin/haemosiderin (30%) mainly in the liver and spleen[1,2,16].

Iron absorption, transport, storage, utilisation, recycling and excretion are mostly genetically controlled by effective regulatory metabolic pathways, homeostatic mechanisms and related proteins[1,2]. A large number of iron containing proteins play an essential role in physiological functions such as oxygen and electron transport, DNA synthesis, food oxidation, drug detoxification, *etc.*[1,2,14,19]. Genetic changes, iatrogenic, nutritional and other factors can affect all the metabolic pathways and physiological functions related to iron and result in iron metabolic abnormalities.

General aspects of iron metabolism have been previously reviewed[1,2,14,19]. Basically, under normal conditions iron is absorbed from the gut then transferred to transferrin in the blood which distributes and delivers iron to the tissues primarily for storage in the liver and utilisation in the production of haemoglobin in the bone marrow. Different but smaller amounts of iron are distributed to other cells and tissues primarily for storage and utilisation for the synthesis of iron containing proteins. Iron from the catabolism of haemoglobin of effete red blood cells is recycled and redistributed by transferrin.

The transport and distribution of iron is tightly controlled. Under normal conditions transferrin is saturated 25%-35% with iron. The intracellular uptake of iron from transferrin and its storage or utilisation in the cells is regulated by the iron regulatory proteins through the translational control of the synthesis of the transferrin receptors at the cell surface and also that of intracellular ferritin. The amount of iron delivered to cells is mainly determined by the number of transferrin receptors and also the iron saturation of transferrin[1,2,14,19].

Cellular iron export is controlled by ferroportin and hepcidin. The latter is an iron-regulatory 25 amino acid peptide hormone produced by the liver. Serum hepcidin concentrations appear to correlate with liver hepcidin mRNA expression, transferrin saturation and nonheme liver iron[1,2,21]. It also appears that hepcidin controls iron export by binding to the protein ferroportin and causing its internalization from the cell surface and subsequent degradation. In general, it is thought that increased liver hepcidin expression decreases the activity of the cellular iron exporter ferroportin. For example in hereditary hemochromatosis, decreased activity of hepcidin in the enterocyte will increase basolateral iron transfer into plasma and consequently cause an increase in dietary iron absorption[22,23]. Hereditary hemochromatosis is mainly caused by a mutation in the *HFE* gene that involves the HFE protein which is predominant in the expression of hepcidin[24]. In contrast, in the anaemia of chronic disease the opposite action, *i.e.,* increased activity of hepcidin in the reticuloendothelial macrophages would decrease iron transfer to plasma and consequently cause

a decrease in the transport of iron to the bone marrow and reduction in haemoglobin production.

In general iron balance in normal individuals is achieved when the rate of dietary iron absorption is equivalent to the rate of iron utilisation and excretion[25]. Iron imbalance can occur due to genetic, regulatory, environmental, iatrogenic and dietary factors. The imbalance is usually related to changes in the rate of iron absorption, utilisation, distribution, excretion, blood loss and intake from transfusions. Iron deficiency for example can occur if the rate of iron absorption is lower than the rate of iron excretion, *e.g.*, nutritional iron deficiency in vegetarians[25]. Similarly, iron deficiency can occur if the rate of the iron utilised, *e.g.*, by the foetus and the mother in pregnancy is higher than the rate of iron absorbed. Another example is the anaemia of chronic disease where iron is diverted and accumulated in the reticuloendothelial system instead of the erythropoietic tissues resulting in anaemia. Blood loss from trauma, haemorrhage and blood donation can also result in iron deficiency anaemia.

In contrast, in iron overload the rate of iron absorption is higher than the rate of iron excretion, *e.g.,* in hereditary haemochromatosis[26,27]. Iron overload can also be caused by regular red blood cell transfusions in conditions such as in thalassaemia, myelodyspasia and sickle cell diseases[7,8,28]. In contrast to the tissue damage observed in hereditary haemochromatosis and thalassaemia, which proceeds progressively for several years, the tissue damage observed in iron poisoning from the accidental ingestion of oral iron preparations is an acute form of iron toxicity and can be fatal in most cases within hours or days unless emergency treatment is provided[29,30].

Overall, many abnormalities exist in relation to body iron balance and distribution, the iron containing proteins and their function and the regulation of the iron metabolic pathways. Many of these iron abnormalities can lead to a number of serious diseases. Within this context, our understanding of the molecular aspects and metabolic pathways related to iron and chelation therapy, as well as other therapeutic interventions can improve therapeutic targeting in diseases of iron metabolism. At the same time misinformation on the iron metabolic pathways may lead to the development of ineffective or potentially toxic therapeutic interventions.

The spectrum of therapeutic interventions in relation to iron metabolism is not limited only to abnormalities of iron metabolism but is extended to many other pathological conditions since iron is playing an important role in the growth and development of all type of cells including normal, microbial and cancer cells. Furthermore iron plays an important role in free radical metabolism and pathology, which is a key factor in tissue damage in almost all pathological conditions[10,11,19].

Simple and inexpensive therapeutic procedures such as iron supplements to treat iron deficiency anaemia and red blood cell transfusions to treat refractory anaemias are widely used. In contrast, venesection is widely used in blood donation and to treat hereditary hemochromatosis[27]. Similarly, erythropoietin in combination with iron is used in the treatment of the anaemia of chronic disease. The therapeutic targeting and interventions can involve many other aspects of the iron metabolic pathways including genetic manipulation, biological therapies using antibodies against regulators, *e.g.*, hepcidin and erythropoietin or antibodies against receptors, *e.g.*, transferrin receptors, *etc*[31,32].

A major role in the development of therapeutic strategies in the treatment of abnormalities of iron metabolism is the design of targeted therapies using iron chelators. Within this context, although the primary therapeutic role of iron chelating drugs is the treatment of transfusional iron overload, many other possible applications of chelators involving all metabolic aspects of iron could be developed. For example the iron chelating drugs DF and L1 could be used in the detoxification of other toxic metals such as aluminium overload, as antioxidants or as antimicrobial agents, *etc*[19,33-35].

THERAPEUTIC APPLICATIONS AND CONTROVERSIES IN THE USE OF CHELATING DRUGS IN IRON METABOLIC DISORDERS

Chelating drugs and chelators could in principle affect and target all the metabolic pathways and proteins involved in iron metabolism either directly through iron binding or indirectly through the intracellular iron pools. They can also affect other metabolic pathways indirectly which are related or influenced by chelation of other metals or related to other aspects of the chelator molecular structure not related to iron[14].

In principle iron chelators can remove, donate and exchange iron, form ternary iron complexes with proteins, other chelators or ligands. They can also be involved in redox reactions mainly with iron and copper and proteins carrying these metals. The chemical, biological, pharmacological and toxicological properties of the chelators are different to those of their iron complex or their metabolites. Chelators have to compete for iron at all the stages of iron absorption, storage, utilisation and excretion with endogenous natural low molecular weight chelators such as citrate, glutathione, ATP, ADP, *etc.*, and also with protein chelators such as transferrin, lactoferrin, haem containing proteins *etc*[14,18,36]. Similarly, the presence of other metals may interfere with chelator iron binding and chelators may affect the metabolic pathways of other metals[37,38]. Overall many interactions can affect the efficacy and toxicity of the chelating drugs *in vivo*[14,36,37].

The mode of action, efficacy and toxicity of the iron chelating drugs DF, L1, DFRA and of other iron chelators are directly related to their physicochemical, pharmacological, toxicological, iron binding and other

Table 1 Property differences and mode of action of chelating drugs

Recommended doses for the chelating drugs in thalassaemia patients
DF subcutaneously 40-60 mg/kg per day; Oral L1 75-100 mg/kg per day; Oral DFRA 20-40 mg/kg per day
Transfusional iron loaded patient compliance with chelating drugs
Low compliance with DF in comparison to oral L1 and oral DFRA
Increase in iron excretion and route of elimination in iron loaded patients
L1: Urinary iron; DFRA: Faecal iron; DF: Urinary and faecal iron
Effect of chelating drugs on iron absorption
Increase of iron absorption by the lipophilc maltol, 8-hydroxyquinoline and DFRA. Decrease of iron absorption by the hydrophilic DF, EDTA, DTPA and L1
Iron removal from diferric transferrin in iron loaded patients
About 40% at L1 concentrations > 0.1 mmol/L, but not by DF or DFRA
Differential iron removal from various organs of iron loaded patients
L1 preferential iron removal from the heart and DFRA from the liver
DF from the liver or heart. (Efficacy is related to dose for all chelators)
Iron redistribution in diseases of iron metabolism by chelating drugs
L1 and to a lesser extent DF can cause iron redistribution from the reticuloendothelial system to the erythron in anaemic rheumatoid arthritis patients. DFRA may cause redistribution of iron from the liver to other organs in thalassaemia and other iron loaded patients. Enterohepatic circulation by DFRA and metabolites
Increase excretion of metals other than iron, *e.g.*, Zn and Al
Order of increased Zn excretion in iron loaded patients: DTPA > L1 > DF
DF and L1 cause increase Al excretion in renal dialysis patients
DFRA causes an increase in Ca excretion and Al absorption (?)
Iron mobilisation and excretion of chelator metabolite iron complexes
Several DF metabolites have iron chelation potential and increase iron excretion but not L1 glucuronide
Chelating drugs minimising other drug toxicity
L1 but not DFRA, inhibit doxorubicin induced cardiotoxicity
Combination chelation therapy
L1, DF and DFRA combinations are more effective in iron excretion than monotherapy. The ICOC L1 and DF combination causes normalisation of the iron stores in thalassaemia patients
Chelating drug synergism with reducing agents
Ascorbate act synergistically with DF but not L1 for increasing iron excretion
Chelating drug antioxidant effects
L1 and DF have shown antioxidant action in *in vitro*, *in vivo* and clinical settings. The antioxidant effects of DFRA are under evaluation

L1: Deferiprone; DF: Deferoxamine; DFRA: Deferasirox; ICOC: International Committee on Chelation; DTPA: Diethylenetriaminepentaacetic acid; EDTA: Ethylenediaminetetraacetic acid.

properties (Figure 1 and Table 1). Within this context the property differences and mode of interactions with different molecular targets are the most important and critical parameters determining the specificity of the iron chelating drugs and also their targeting profile for the treatment of iron overload and other diseases (Table 1)[14,36].

The primary use of the chelating drugs is the treatment of iron overload in thalassaemia and other transfusional iron loaded conditions. Iron overload toxicity from chronic transfusions involves multi-organ damage and low life expectancy. In the absence of chelation therapy thalassaemia patients die by the age of 20 years, mainly from congestive cardiac failure caused by cardiac iron overload toxicity[5,7,8].

There are big differences in the efficacy, tolerance, site of action, toxicity profile and the cost of the chelating drugs, which affects the morbidity and mortality of thalassaemia patients both in developed and developing countries (Table 1)[5,7,8].

There are also general variations among patients in response to each chelating drug, which is related to their differences in the absorption, distribution, metabolism, elimination and toxicity[5,9,39-41].

The recommended doses for the chelating drugs in thalassaemia are 40-60 mg/kg per day for subcutaneous DF, 75-100 mg/kg per day for oral L1 and 20-40 mg/kg per day for oral DFRA. Compliance is low with subcutaneous DF in comparison to oral L1 and DFRA. The site and level of iron removal is different among the chelators with L1 being the most effective in iron removal from the heart resulting in an increase in life expectancy in thalassaemia patients that have been using it in the last two decades[9,42]. In contrast, high morbidity and mortality have been reported in different categories of patients that have been treated with DFRA[43,44]. The efficacy of iron removal from thalassaemia patients by DFRA is lower than DF or L1, especially regarding iron removal from the heart[45]. The most effective treatment of cardiac iron overload are selected combinations of L1 and DF[46].

Many of the controversies in the use of chelating drugs arise from the different influences and priorities for use by the regulatory authorities, clinicians and patients[45]. For example, there is no consensus in the ultimate goal or aim of the chelation therapy in thalassaemia and other transfusional iron loaded conditions or the selective use of each of the chelating drugs for optimal therapy. There is also no consensus in the evaluation criteria and risk/benefit assessment for the use of each of the chelating drugs in personalised medicine[47]. In most countries the selection of the chelating drug for the treatment of iron loaded patients depends on the commercial influence of pharmaceutical

companies[5]. The situation regarding the use of the chelating drugs in the developing countries where most patients live is not only concerning issues related to the risk/benefit assessment but mainly issues regarding their availability and cost. Such issues have been recently highlighted within the broad context of the use of orphan drugs in orphan and rare diseases which includes thalassaemia and other transfusional iron loaded conditions[5].

Recent developments involving mainly clinical findings and the application of new diagnostic techniques such as magnetic resonance imaging (MRI) T2 and T2* has increased our understanding of iron metabolic and chelation pathways of iron removal and resulted in improved drug targeting therapies of iron toxicity[48-50]. These developments increased the prospects of the introduction of personalised medicine in thalassaemia and other iron metabolic disorders. Based on these findings the complete treatment of iron overload and reduction of morbidity and mortality in thalassaemia using L1 or the L1/DF combination has been recently achieved[9].

Similarly, recent developments involving the prospect of wider use of chelating drugs and in particular of L1 as a universal antioxidant in non iron overload diseases such as neurodegenerative, cardiovascular, renal, infectious diseases as well as other diseases including cancer and ageing has been investigated in clinical trials and within the broad context of the risk/benefit assessment because of the absence of other effective therapeutic approaches and developments in many of these conditions[9,36,51].

The introduction of L1 for the treatment of non iron loaded patients by targeting focal toxic iron deposits, *e.g.*, in Friedreich ataxia and toxic labile iron, *e.g.*, in diabetic and non-diabetic glomerular disease is a reflection of the antioxidant and safety potential of this drug[10,11,19]. The safety of L1 in many categories of non iron loaded diseases has also been confirmed in clinical trials involving patients with the anaemia of chronic disease, renal dialysis, infections, Parkinson's and other neurodegenerative diseases, *etc*[10,11,19]. As in many other cases of drug development the introduction prospects of L1 in these diseases is based on commercial and not ethical criteria[5].

CONTROVERSIES REGARDING MOLECULAR ASPECTS OF IRON METABOLIC DISORDERS AND CHELATOR INTERVENTION

Normal iron metabolism is generally characterised by the normal function, pathways and activity of iron containing proteins including physiological levels of haemoglobin, serum ferritin, serum iron, serum transferrin saturation, liver and other organ iron store levels, *e.g.*, those estimated by MRI, *etc*[48-50]. These physiological levels are the main regular parameters measured in clinical laboratories and MRI units for the identification of iron overload and other metabolic abnormalities.

Many of the disease models related to iron metabolic abnormalities appear in general to be affected by genetic, regulatory and iatrogenic factors. However, like in all other diseases there are different levels of pathological and compensatory mechanisms working in parallel with the main disease pathways and mechanisms. Similarly, there are also many other factors such as dietary, pharmacological and environmental factors that can influence or supersede the normal pathways and affect the levels of iron, as well as the prognosis and treatment of patients with iron abnormalities.

Some compensatory mechanisms of limited impact observed in beta thalassaemia are related to the variation of the age range of survival of non transfused patients. In these cases despite the absence of the production of normal haemoglobin (HbA) the survival is not uniform and can range from 1 to 7 years. The difference in the survival age among this group of patients appears to be related to a number of factors. For example beta thalassaemia patients producing higher levels of foetal haemoglobin (HbF) have increased survival prospects and agents inducing the production of HbF are the subject of clinical investigations and development for the treatment of beta thalassaemia[52,53].

Another compensatory mechanism in iron metabolism is observed during venesection in hereditary haemochromatosis and blood donation where stored iron mainly originating from the liver is steadily transported to the bone marrow for restoring iron balance and the normal production of haemoglobin.

Variations in the progression of neurodegeneration, cardiomyopathy and other toxic side effects observed among Friedreich Ataxia patients is thought to be related to the production of the protein frataxin and many other factors influencing the rate of accumulation and toxicity of iron in mitochondria[54,55].

Many other iron regulatory and compensatory mechanisms operate under normal conditions and iron metabolic disorders. One major intervention mechanism or pathway that can supersede many regular pathways and can affect many diseases of abnormal iron metabolism is targeted chelation therapy. Within this context, most physiological process related to iron can be affected including iron absorption, excretion and delocalisation[14,36].

MECHANISMS OF IRON ABSORPTION AND THE INFLUENCE OF CHELATORS

Body iron intake under normal conditions is mainly controlled by the rate of iron absorption and the rate of iron turnover in the bone marrow for the production of haemoglobin and red blood cells. In considering the iron absorption mechanisms the main classical pathway is thought to involve the iron uptake from the gut lumen

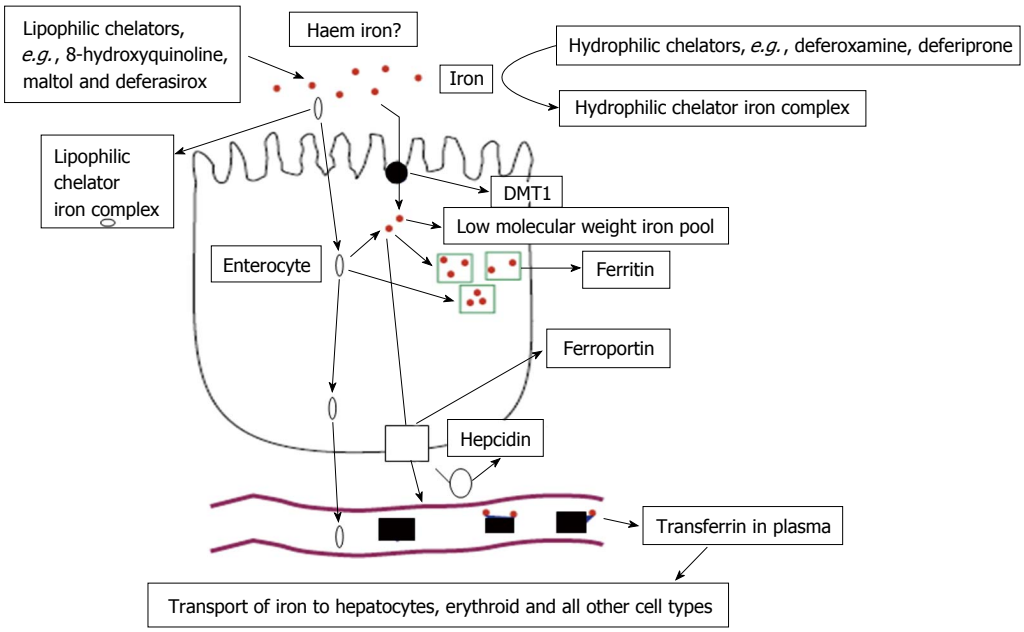

Figure 2 Iron absorption mechanisms at the enterocyte. Under normal conditions the regulatory pathway of iron absorption at the enterocyte involves the regulatory molecules DMT1, hepcidin, ferroportin and then iron transfer and uptake by transferrin in plasma. A parallel pathway of iron absorption may involve lipophilic dietary chelating molecules like maltol. Different pathway of iron uptake by the enterocyte also exists for haem iron. Adapted from ref. [22]. DMT1: Divalent metal transporter 1.

by the enterocytes using the Dcytb and divalent metal transporter 1 pathway, incorporation intracellularly into the low molecular weight iron pool and ferritin. Iron within the enterocyte is then thought to be partially exported *via* the regularly controlled ferroportin/hepcidin pathway, oxidation of iron by hephaestin and lastly uptake by transferrin in plasma for distribution to all cell types of the body and in particular the hepatocytes for storage and the erythroid cells for the production of haemoglobin (Figure 2)[1,2].

Despite that this may appear to be the main iron absorption pathway under normal conditions there are clinical and laboratory evidence of alternative independent mechanisms operating at different levels (Figure 2)[22]. Clinical evidence for the operation of alternative pathways of increased iron absorption which supersedes the main mechanism is observed in the use of iron supplements and food fortification, also in Bandu siderosis where excess iron is absorbed from iron pots used for cooking and lastly in acute iron poisoning from the accidental ingestion of tablets or other oral iron formulations[25,29,30]. In all the above cases the presence of increased quantities of iron in the gut results in excess iron absorption, transport and deposition in the body[22,25]. It appears from these and also other cases that the rate of iron absorption partly depends on the quantity of iron present in the gut[25,26].

In addition to the quantity, the quality of iron presented in the gut lumen is another determining factor affecting iron absorption with ferrous and haem iron being more readily absorbed than ferric iron (Figure 2)[22,25,26]. Another, more effective pathway that supersedes the main pathway and causes substantially higher amounts of iron absorption is lipophilic iron chelator complexes including different haem compounds, which may have a use in the treatment of iron deficiency anaemia (Figure 2)[22]. For example the long term oral administration of the lipophilic chelator 8-hydroxyquinoline caused iron overload in animals and also oral administration of several lipophilic iron complexes such as those of 8-hydroxyquinoline, 2-hydroxy-4-methoxypyridine-1-oxide and maltol caused several fold increases of iron absorption in comparison to animals used as controls (Figures 1 and 2)[56,57]. Maltol in particular, was originally identified as a chelator intended for clinical use in iron deficieny at the same time that L1 was identified for the treatment of iron overload[58]. Maltol also caused increased iron absorption in several clinical trials and in particular it reached phase Ⅲ clinical trial stage in patients with iron deficiency anaemia with inflammatory bowel disease[59,60].

In contrast to lipophilic chelator iron complexes, chelators forming charged hydrophilic iron complexes such as DF and L1 or chelators causing iron precipitation such as phytates and tannins appear to decrease iron absorption and may have a use in the treatment of thalassaemia intermedia and hereditary haemochromatosis[22,57]. Similarly, chelators inhibiting iron absorption and the prevention of iron uptake by the cancer cells of the colon may have a preventative and therapeutic use in the iron induced colorectal cancer[61]. It is envisaged that overall many naturally occuring dietary compounds and medicinal drugs with chelating properties will affect iron absorption in a manner similar to that observed by lipophilic and hydrophilic chelators[18,25].

Another controversial issue in the mechanism of iron absorption which is also promoted in textbooks for cellular iron export is the suggestion of the presence or

need of an oxidation pathway for iron by hephaestin or caeruloplasmin before iron chelation by transferrin. This process and suggested pathway is questioned since transferrin has strong ferroxidase activity similar to the chelating drugs L1 and DF, oxidising Fe (Ⅱ) to Fe (Ⅲ) before chelation and ferric complex formation. In fact, the ferroxidase and iron binding activity of transferrin is one of the most effective and efficient antioxidant systems operating in blood plasma and no mediator protein is required or envisaged to participate in this process[62,63].

In addition to chelator iron uptake and transfer pathway by transferrin, many other pathways and mechanisms are thought to operate in parallel with the main proposed mechanisms. It should be noted for example that even in the case of the rare disease atransferrinaemia, iron is absorbed and finds its way to the liver and the erythropoietic tissues, suggesting that a compensatory mechanism is in operation in addition to transferrin for iron transport in blood and supply to the tissues[64]. Although this secondary pathway is not as efficient and leads in the long term to iron toxicity, the mechanism operating is not clear but resembles or is related to another controversial issue of iron metabolism namely NTBI.

The formation and potential toxicity of NTBI has been previously discussed and reviewed with different opinions on the impact on iron overload and other diseases[62,63,65,66]. Almost all thalassaemia patients with serum ferritin greater than 500 μg/L appear to have fully saturated transferrin and different amounts of NTBI[62,63,65]. Despite that there is evidence of oxidative stress toxicity caused by NTBI in iron overloaded thalassaemia, hereditary haemochromatosis and other categories of patients, there is no evidence that the level of toxicity by NTBI is sufficient to cause tissue damage. In contrast, the level of excess deposited iron and especially of haemosiderin iron is considered the main cause of tissue damage and organ toxicity (*e.g.*, heart, liver, pancreas, *etc*.) in iron overload in thalassaemia and other conditions[48-50,67].

Another controversy in relation to the NTBI toxicity in clinical practice is the regulatory health authorities approved administration of intravenous iron which is widely and routinely used in renal dialysis, inflammatory bowel disease and many other categories of anaemic patients. The amount of NTBI formed during intravenous iron is much higher than thalassaemia or other iron loading conditions but no permanent or serious iron related toxicity has generally been reported[68,69].

MECHANISMS OF IRON EXCRETION AND THE INFLUENCE OF CHELATORS

Iron excretion is a major area of iron metabolism, which however is generally neglected in comparison to iron absorption and other pathways of iron physiology. The mechanisms and pathways of iron excretion and iron loss as well as their implication on the body iron status have been previously reviewed[25,70]. Despite the fact that the presence of a regulatory iron excretion model has not yet been fully explored, such a pathway plays an important role in iron balance. For example, iron deficiency anaemia in adults under normal conditions can only be manifested if the rate of iron excretion or loss is higher than the rate of iron absorption[25,70].

In general several factors such as the body iron load, plasma iron concentration, physical activity, infections, pathological conditions and dietary habits affect the level of iron excretion[25]. The presence of regulatory iron excretion is also supported by other clinical findings such as the slow but steady reduction in the iron load of transplanted ex-thalassaemia patients in the absence of chelation or venesection[71,72].

The concept of iron excretion is mostly highlighted in studies involving iron chelation therapy in conditions of iron overload and also in iron balance studies of non iron loaded conditions. In the latter cases there have been reports of decrease in haemoglobin levels following treatment using L1 for several months, *e.g.*, in Friedreich ataxia patients[73]. In iron overload the level of iron excretion generally depends on the chelating drug and the dose used and also the body iron load of the patients[74]. The iron pools affected during the iron mobilisation and the routes of excretion (faecal and or urinary) vary among the chelating drugs and other chelators[14,25,47]. In the case of L1 iron is excreted almost exclusively in the urine, DFRA is almost exclusively in the faeces and DF mostly in the urine and some in the faeces (Table 1)[14,25].

The efficacy in iron mobilisation of excess stored iron from the organs of iron loaded thalassaemia patients is different among the chelators used with L1 being the most effective in the mobilisation of excess iron from the heart, DF less effective and DFRA the least effective. In contrast DF and DFRA appear to be more effective in the mobilisation of iron from the liver than the heart[45,46,50,74,75]. In most clinical trials studying the efficacy and effects of iron removal by chelating drugs in iron loaded thalassaemia patients the results are inconclusive because of the use of different dose or range of doses[74].

The most effective chelation treatment leading to the complete normalisation of the iron stores in iron loaded thalassaemia patients is the combination of L1 and DF (Figure 3)[76-80]. Specific dose protocols have to be used for this purpose, for example the International Committee on Chelation (ICOC) protocol which consists of daily oral L1 at 75-100 mg/kg per day and subcutaneous DF at 40-60 mg/kg at least 3 d/wk[77]. Thereafter monotherapy of L1 at 50-100 mg/kg per day is sufficient in most cases for maintaining normal range body iron store levels[78,80].

Many naturally occuring iron chelators present in food, usually of plant origin are expected to affect the rate of iron absorption and excretion in a mode of action

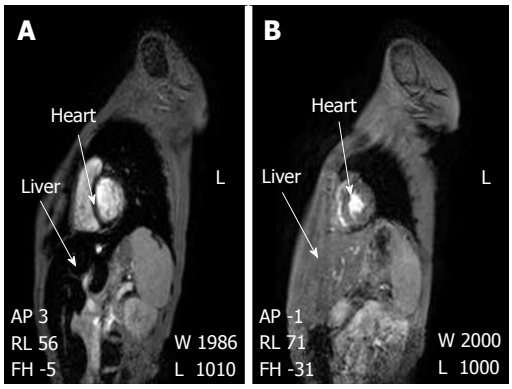

Figure 3 Clearance of iron overload of the liver and heart of a thalassaemia patient using the deferiprone deferoxamine combination. The MR image changes before (A) and after (B) the L1/DF combination therapy. Short axis view of liver and heart of a thalassaemia patient at 4 mo before the L1/DF combination (A: Cardiac T2* was estimated as 9.3 ms and liver T2* as 3.8 ms. The serum ferritin was 727 μg/L, 2.5 mo before the MRI scan) and 9 mo after the combination (B: Cardiac T2* was estimated as 23.0 ms and liver T2* 26.2 ms. The serum ferritin was 166 μg/L, 0.5 mo after the MRI scan). Arrows indicate the liver and interventricular septum of the heart, respectively. Adapted from ref. [74]. MRI: Magnetic resonance imaging; L1: Deferiprone; DF: Deferoxamine.

similar to that described by lipophilic and hydrophilic chelators. Within this context under normal conditions naturally occuring chelators with similar properties to the chelating drugs L1, DFRA and DF are expected to increase iron excretion and affect the overall body iron balance[25]. The chelating efficacy of naturally occuring chelators is concentration dependent and in most cases low and may act synergistically with other chelators or the chelating drugs in iron mobilisation.

On the molecular level iron mobilisation by chelators is thought to proceed at different rates from the available chelatable pools with NTBI to be readily and instantly available by comparison to transferrin iron which is only available to L1 and can take about 1 h to reach completion *in vitro*[58,81]. The reaction is L1 concentration dependent and partial transit de-ironing from transferrin is observed in the serum of iron loaded thalassaemia patients[58,62,63,81-84].

In the intracellular iron mobilisation by chelators, the transit low molecular weight iron pool is readily available followed by haemosiderin and then ferritin iron[85]. The reaction is chelator concentration dependent and takes 2-3 d to reach completion[86]. In the iron mobilisation from ferritin the first in last out principle of iron removal operates. Less iron removal is observed by L1 and other chelators with ferritin molecules containing smaller iron cores in comparison to ferritin molecules containing larger iron cores[87]. It appears that there is lower exposure of the surface iron core to chelators by comparison to larger iron cores[87]. It was also observed that the solubility and mobilisation of iron by chelators increases in ferritin and haemosiderin with newly formed more hydrated oxohydroxy iron cores in comparison to ferritin and haemosiderin with less hydrated older cores of iron oxohydroxy bridges[85,87].

Mobilisation of iron by L1, DF and other chelators from other iron containing proteins, *e.g.*, haemoglobin has not been shown[88]. Exception was lactoferrin where iron removal by chelating drugs has only been shown in the case of L1[89].

CONDITIONS WITH ABNORMAL IRON DEPOSITION AND THE RELOCATION OF IRON BY CHELATORS

Under normal conditions iron is considered to be uniformly distributed in the various organs. In hereditary haemochromatosis the storage of excess iron is primarily in the parenchyma cells of the liver. The storage of excess iron in transfusional iron conditions is mostly in the parenchyma and Kuppfer cells cells of the liver, spleen and cardiocytes.

Until recently it was believed that in transfusional iron overload in thalassaemia, iron was uniformly distributed in the various organs and also that serum ferritin and liver iron reflected body iron store levels. However many clinical findings and iron load estimations using MRI T2 and T2* suggests that serum ferritin is in most cases only related to liver iron stores but not to spleen, heart and pancreas iron load[50,90-93]. It was also observed using MRI that in many thalassaemia patients the liver is overloaded with iron but the heart has normal iron range levels. In contrast, in some thalassaemia patients the reverse is true, i.e., the heart is overloaded with iron but the liver has normal iron range levels (Figure 4)[14,90,93]. This last finding provides an explanation for many of the fatal cases of thalassaemia patients prior to the introduction of MRI, who died from congestive cardiac failure despite very low serum ferritin and liver iron concentration. Within this context, the prophylactic use of L1 is essential for preventing cardiac damage[94,95].

The role of spleen as a major iron storage organ, sometimes of equal importance to liver iron storage and also in the ferrikinetics of iron overload in thalassaemia patients was highlighted in a number of studies (Figure 5)[96,97]. Despite that an increase in haemoglobin was expected following splenectomy in thalassaemia patients the substantial increase in serum ferritin provided further evidence that serum ferritin is not related to total body iron load but mostly to the concentration of stored iron in the liver[95]. Furthermore, following splenectomy excess iron may be diverted to the heart causing myocardiac iron loading and cardiomyopathy[97].

In general, it appears that serum ferritin and liver iron estimations are misleading regarding cardiac and other organ iron load as well as total body iron load in thalassaemia patients[93]. MRI T2 and T2* findings also appear to suggest that in many cases of iron loaded thalassaemia patients the deposition of iron in the liver, spleen and heart is not uniformly distributed within each organ[50]. These mosaic iron distribution of dense and light iron deposits in the liver and heart was particularly evident during the normalisation of the iron stores of

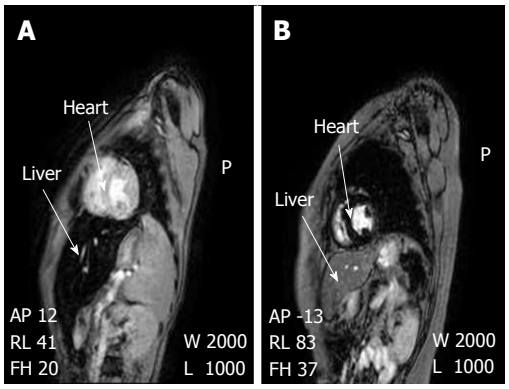

Figure 4 Non homogeneous iron distribution among the organs of iron loaded thalassaemia patients. Differential iron loading of the heart and liver of two iron loaded thalassaemia patients using MRI and T2* estimation. A: Heavy haemosiderosis of the liver [T2* = 1.2 ms (normal T2* ⩾ 6.3)] and normal T2* of the heart (T2* = 20.6). The top arrow shows the interventricular septum of the heart of the patient with no iron deposition (normal) where the bottom arrow shows the heavy iron loading within the liver parenchyma, demonstrated as low signal intensity (dark); B: Heavy haemosiderosis of the heart (T2* = 6.32 ms) and normal T2* of the liver (T2* = 19.2 ms). The top arrow shows the abnormal iron deposition in the interventricular septum of the heart of the patient, which is shown with low signal intensity (dark). The bottom arrow shows the liver of the patient with no iron deposition (normal). Adapted from ref. [14]. MRI: Magnetic resonance imaging.

thalassaemia patients treated with the L1/DF ICOC combination protocol[50]. Similar findings of non uniform iron distribution are observed in liver and spleen biopsies (Figure 5). These findings provide an explanation for the high level of error of liver biopsies for estimating iron load which was previously observed in many studies with thalassaemia patients.

There are many acquired and hereditary conditions with abnormal iron distribution leading to body iron imbalance and in many cases specific tissue iron localisation and anaemia. In the anaemia of chronic disease iron is mostly stored in the cytoplasm of reticuloendothelial macrophages. This form of anaemia is observed in many chronic inflammatory and other conditions such as rheumatoid arthritis, chronic kidney disease and cancer[98,99]. It is believed that in these and other conditions there is an increased production of hepcidin and decrease in the ferroportin activity of the reticuloendothelial macrophages. These changes cause a decrease in iron transfer from the reticuloendothelial macrophages into plasma and subsequently reduction of iron availability to the bone marrow, reduction in haemoglobin production and consequently anaemia[1,2,98,99].

A similar mechanism of increased hepcidin production leading to plasma iron reduction is thought to operate in the hypoferremia of infectious diseases. This mechanism appears to reduce transferrin bound iron and iron bioavailability to the siderophores of microbes restricting their growth[100,101]. This mechanism is important for iron loaded patients who are more susceptible to siderophilic bacteria infections and have increased incidence of morbidity and mortality associated with infections[35,102]. A hepcidin independent pathway for the hypoferraemia in infections has also been identified[103]. Within this context pharmacologic modulation of iron metabolism and chelation therapy may be potential strategies to control infection[35,63].

There are many other diseases of abnormal iron deposition which originate from inherited, environmental, iatrogenic and metabolic factors with different health implications. For example increased iron accumulation and deposition is observed in mitochondria in sideroblastic anaemia and Friedreich Ataxia but not in the mitochondria of iron overloaded thalassaemia or hereditary haemochromatosis patients[54,55,95,104-106]. Furthermore, despite that iron is also diverted and causes mitochondrial iron deposition and anaemia in sideroblastic anaemia patients, in general no anaemia or abnormal serum iron or serum ferritin levels are observed in Friedreich Ataxia patients[9,107-109].

The localisation of focal deposited iron in the brain has been recently identified by MRI in many neurodegenerative and other diseases such as Friedreich Ataxia, Parkinson's and Alzheimer's diseases and Hallevorden-Spatz syndrome[110-115]. However, a major difference between the above conditions and iron overloaded thalassaemia patients is that in the latter group of patients there is no iron accumulation in the brain or related toxic side effects involving the nervous system.

Chelation therapy could be introduced in many of the abnormally localised deposited iron conditions described above by bypassing the related mechanisms and may lead to the correction of the abnormality. Such intervention may restore iron balance, eliminate the associated iron toxicity or reduce the anaemia. Within this context a number of clinical trials were carried out using chelating drugs in different categories of patients where iron was not normally distributed.

In one study the effect of L1 chelation therapy was investigated in the anaemia of chronic disease using a group of anaemic rheumatoid arthritis patients including some not responding to erythropoietin. The patients were treated with L1 up to 2 × 2 g/d for a week. A substantial increase in haemoglobin levels were observed at the end of the study[116,117]. The mechanism operating in this group of patients treated with L1 was thought to involve several stages. In the initial stage, the mobilisation of stored iron by L1 from different sites including the reticuloendothelial macrophages was anticipated as previously shown with *in vitro* macrophage cell studies[118]. In the subsequent stage, the iron mobilised by L1 was thought to be partly donated to unsaturated transferrin increasing transferrin iron saturation as previously shown with *in vitro* studies and the 7 h progressive increase in transferrin iron saturation of up to 80% in normal volunteers treated with L1[83,119]. In the last stage iron saturated transferrin increases the transfer of iron to bone marrow and other erythropoietic tissues causing an overall increase in the production of haemoglobin[116,117].

These studies suggest that the chelation pathway may compete and override the hepcidin and erythropoietin

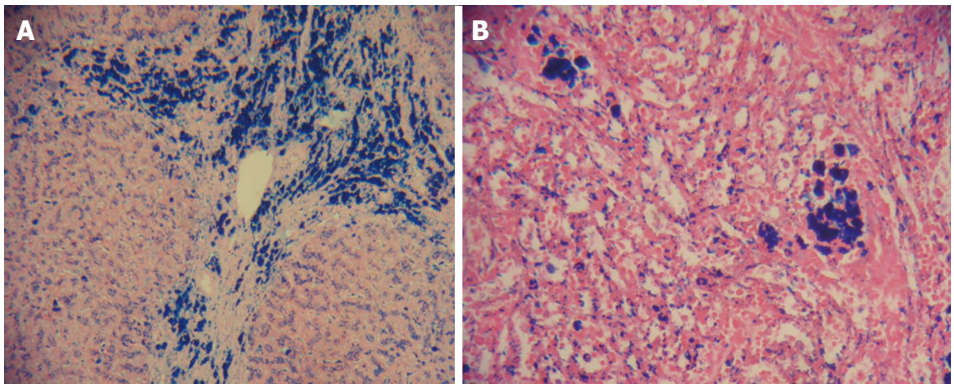

Figure 5 Non homogeneous iron distribution in the liver and spleen of an iron loaded thalassaemia patient. Liver and spleen biopsy photographs (× 20) of a 29-year-old, 55 kg male thalassaemia patient. The liver biopsy was obtained during splenectomy. A: Liver section showing non unifom iron deposition stained with Pearl's Prussian blue. There are hemosiderin deposits in hepatocytes and Kupffer cells and especially within bile ducts; B: Spleen section where iron deposits were stained with Pearl's Prussian blue. There are non uniform hemosiderin deposits within cytoplasma and nucleus of macrophages. Four months before the splenectomy the patient had an MRI T2* (ms) of heart 4.1, liver 0.0, spleen 2.9, and serum ferritin of 3850 μg/L. Adapted from ref. [96]. MRI: Magnetic resonance imaging.

pathways in the anaemia of chronic disease.

Similar results of focal iron deposit removal and relocation was observed in other diseases involving different organs. Iron removal from focal iron deposits in the brain has been shown using L1 in a number of clinical trials involving Friedreich Ataxia patients. In one study nine Friedreich ataxia patients were treated with 20-30 mg/kg per day of L1 for 6 mo. Substantial reduction of the stored toxic iron in the brain was diagnosed using MRI T2* following L1 treatment, which coincided with a reduction in ataxic gait and neuropathy[120]. Similarly, neurological and heart function benefits were identified in further L1 trials in Friedreich Ataxia and other patients[121-124].

Iron toxicity derived from focal or labile iron deposits has also been implicated in the tissue damage of many other diseases. Targeted chelation therapy was also used to prevent or minimize such toxicity. For example encouraging therapeutic results were observed in clinical studies involving about 50 non iron loaded patients with acute kidney disease using L1 at doses of 50-75 mg/kg per day for up to 9 mo[125]. No serious toxic side effects were reported during the studies in this category of patients and L1 was shown to improve kidney function and to cause a decrease in proteinuria[125].

The use of iron chelating drugs in many other conditions such as infections, inflammation, cytotoxic therapies, detoxification of other metals, drug toxicity as well as many other conditions involving proteins and pathways of iron metabolism is currently in progress[9,14,19,22,63]. However, many therapeutic developments are almost exclusively based on commercial and not ethical considerations[5,126-130]. Furthermore the impact and significance of academic findings in relation to therapeutic developments and their applications in medicine is the subject of selective promotion by editorial boards of medical journals most of which are commercial organisations, with commercial connections and interests.

THE ROLE AND CONTROVERSIES OF MEDICAL JOURNALS IN SHAPING MEDICAL OPINION IN IRON METABOLISM AND CHELATION THERAPY

The lucrative revenues of pharmaceuticals which only for the world's twelve richest pharmaceutical companies based in the United States and Western Europe are estimated at 0.5 trillion United States dollars annually, depend on marketing policies and "lobbying" procedures involving physicians, journals, regulatory authorities, patient organisations and other groups[5,131-134]. Within this context there are many grey areas and conflicts of interests regarding the role of pharmaceutical companies and their influence on government, academia, medical journals and many other organisations or institutions[5,131-135].

Medical journals are major contributors in the dissemination of basic and clinical science information which is used to guide physicians and health professionals in the selection of therapeutics, which are important for the patients' treatment, safety, morbidity and mortality. Most of the clinical trials on the effects of therapeutics published by medical journals are authored by academics founded or sponsored by pharmaceutical companies[135]. Similarly, despite that most members of editorial boards and referees of medical journals are affiliated to academic institutions, the commercial influence on academia and in particular the medical journals are increasing. Most publications related to new patented drugs are usually biased in relation to efficacy and safety and are controlled by medical writers affiliated to the pharmaceutical companies[5,131-135]. Such information is recycled with repeated publications and citations of only positive results, which are attributed to only authors collaborating with the pharmaceutical companies.

The role of leading medical journals which are based

in Western Europe and North America in providing unbiased information on new patented drugs is also questioned, since almost all such journals are businesses and dependent on income from the pharmaceutical industry including advertisements, page charges, reprints, conferences, *etc*[5,131-135]. Such journals are leading in the marketing promotion efforts of multinational pharmaceutical industry of new expensive patented products which sometimes are less safe or efficacious than generic drugs. Such promotions are considered to serve also the national interest of both the pharmaceutical industry and medical journals since the lucrative income from new patented drug sales are major contributors to the economy of the developed countries involved. However, these efforts in many cases undermine the safety and therapeutic outcome of many categories of patients because of inaccurate risk/benefit assessments and questionable clinical benefits made by physicians, *e.g.*, in the use of chelating drugs[5].

Within this context some controversial cases of risk/benefit assessments have been previously identified and reported during the marketing drive and promotion of the use of chelating drugs in relation to the treatment of thalassaemia and other conditions[5,43,44,136-140]. In particular the promotion and use of DFRA in hereditary haemochromatosis and ex-thalassaemia transplanted patients instead of venesection raises major ethical questions. Similar questions have been raised in the risk/benefit assesment of the use of DFRA in thalassaemia intermedia instead of L1 or DF[22,138]. Furthermore, many clinical investigators have also questioned the therapeutic benefits from use of DFRA or of other chelating drugs in myelodyspasia and sickle cell anaemia patients[141,142].

One major controversial issue that led to exchanges between the pharmaceutical company marketing DFRA and an author questioning the safety of the use of DFRA in non iron loaded patients was highlighted in the journal *Lancet* and *Expert Opinion in Drug Safety*[43,44,143,144]. While the exchanges were published in the last journal only the pharmaceutical company's view were published in the *Lancet*, overturning the Journal's rules of submission of correspondence including the length and timing of submission. The issue was raised in a letter to the *Lancet* editors asking among other for the declaration of the commercial links of the journal but the letter was not published. Furthermore the same issue and the favouritism for the company marketing DFRA was raised with the Lancet ombudsman, who indicated that he will investigate the issue but for more than two years is still under investigation and no reply was provided nor the *Lancet's* commercial links declared.

Similar issues in relation to chelating drug development were raised with the journal *Annals of Neurology* regarding the use of L1 in Friedreich ataxia patients where the lack of crucial diagnostic and therapeutic outcome procedures in relation to focal iron levels and lack of iron balance studies were questioned[73]. The need for personalised medicine was also raised since there is wide variation in the severity of the disease and level of focal iron deposits in the heart and brain of Friedreich ataxia patients. In this case the editors of the journal referred to "expensive studies to track iron scores" and "the company developing the drug spends millions of dollars". It should be noted that the original proposal for the use of L1 in Friedreich ataxia patients was suggested many years ago and L1 was developed following academic initiatives[5,36].

Commercial and academic conflicts in relation to L1 development are widely published in the medical literature since its discovery[5]. Most of the academics involved in such conflicts were financed directly or indirectly by competing pharmaceutical companies and not related to independent assessment on drug safety and efficacy[5]. Similarly, the implications of drug costs and drug availability to patients especially in developing countries, including that of the iron chelating drugs or other orphan drugs is rarely discussed or highlighted in medical journals[5].

There are many other issues in relation to the role played by medical journals in shaping medical opinion on drug use and development including that of iron chelating drugs. Such issues are many and vary. For example in most publications the ultimate aim of iron chelation therapy, which is the normalization of the iron stores of regularly transfused patients is avoided or not specified[5]. Similarly, the background history and information regarding drug assessment is not thoroughly investigated by the journal editors or specified in future publications even in the same journal. In one case a clinician reported liver toxicity in thalassaemia patients treated with L1 which was not confirmed by any other investigator[5,145,146]. This case reached the mass media and delayed the development of L1 but it may have caused the life of thousands of patients from cardiac failure[5,145,146].

Several other controversies are overlooked in publications related to chelating drug efficacy and development which affect patient safety and therapeutic outcomes. In many cases comparative therapeutic assessments are carried out in clinical trials using different dose protocols of the iron chelating drugs[5]. Similarly some journals overemphasize the importance of diagnostic techniques such as liver iron estimations or of NTBI, which are not critical for the prognosis of thalassaemia patients and other iron overloaded conditions in comparison to cardiac MRI T2* and T2[45,46,65,67]. This issue partly diverts attention from the difference in the ability of chelating drugs in the mobilisation of iron from the heart[12]. Within this context even the assessment of cardiac iron using MRI T2* was questioned when L1 was shown to be superior to DF in the removal of iron from the heart[147-149].

Many medical journals express their medical preferences for selecting articles based only on the opinion of clinical and other investigators associated with pharmaceutical companies, while ignoring any other authors opinion and any new developments for example in the area of chelation[150-152]. The influence of medical journals

is also highlighted by the submission of publications of clinical investigations to the regulatory authorities. For example this resulted in the difference of timing in the regulatory approval of L1 first in India in 1994, then the European Union in 1999 and lastly in the United States in 2011[153]. Another example is the generic chelating drug EDTA which despite its approval about 50 years ago for metal detoxification it has been used ever since by millions of patients as alternative medicine for many conditions (Figure 1)[153-155]. It is only recently that the health authorities in the United States took an interest on its therapeutic properties in cardiovascular and other conditions[155,156].

Many future studies could be performed to elucidate further and improve the role of chelating drugs in iron metabolism and generally in health and disease. For example, the antioxidant role of chelating drugs used as monotherapy or in combination therapies with other antioxidants could be envisaged in different inflammatory conditions[19,157]. Similarly, the use of iron metabolism indices and algorithms could be introduced in different clinical conditions in order to best evaluate iron deficiency or overload and accordingly adapt iron chelation or iron supplementation and other related therapies[158].

CONCLUSION

Iron metabolic disorders affect more than a quarter of the world's population with a different range of health implications and rates of morbidity and mortality. Iron deficiency anaemia is a major health hazard found mainly in developing countries but can be relatively easily treated using iron supplements or lipophilic chelator iron complexes. Similarly, hereditary haemochromatosis can be easily treated using venesection. In contrast, iron overload in transfusional iron overload for example in thalassaemia is fatal unless chelation therapy is introduced. In most cases L1 in combination with DF and L1 monotherapy can completely treat iron overload in thalassaemia. Deferiprone has also been shown to minimise the toxic effects of pathological iron found in neurodegenerative, renal and other diseases. Deferasirox is more toxic than L1 and DF and can mainly be used in patients not tolerating L1, DF or their combination. Controversies in the risk/benefit assessment for the use of DFRA in thalassaemia, other iron overloaded and non iron overloaded conditions appear to involve commercial interests, and influence of academic medical journals and physicians.

In addition to iron overload many other abnormalities related to iron metabolism and toxicity can be treated using chelators. In particular, iron toxicity is a major factor in free radical pathology and tissue damage in many diseases. Iron chelating drugs can correct iron imbalance for example in the anaemia of chronic diseases and can also minimise iron toxicity related to proteins or pathways of iron metabolism.

The role of medical journals in shaping medical opinion and updating biochemical and clinical findings including issues relating to the risk/benefit assessment of drugs as well as drug safety and efficacy are crucial for patient survival, morbidity and mortality. Many controversies in relation to drug development and use with emphasis the iron chelating drugs are widely reported in the medical literature. Within this context commercial influence and contacts of the medical journals with the pharmaceutical industry and other commercial or government organisations should be declared.

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EDITORIAL

Cation-exchange high-performance liquid chromatography for variant hemoglobins and HbF/A2: What must hematopathologists know about methodology?

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Abstract

Cation-exchange high-performance liquid chromatography (CE-HPLC) is a widely used laboratory test to detect variant hemoglobins as well as quantify hemoglobins F and A2 for the diagnosis of thalassemia syndromes. It's versatility, speed, reproducibility and convenience have made CE-HPLC the method of choice to initially screen for hemoglobin disorders. Despite its popularity, several methodological aspects of the technology remain obscure to pathologists and this may have consequences in specific situations. This paper discusses the basic principles of the technique, the initial quality control steps and the interpretation of various controls and variables that are available on the instrument output. Subsequent sections are devoted to methodological considerations that arise during reporting of cases. For instance, common problems of misidentified peaks, totals crossing 100%, causes of total area being above or below acceptable limits and the importance of pre-integration region peaks are dealt with. Ultimately, CE-HPLC remains an investigation, the reporting of which combines in-depth knowledge of the biological basics with more than a working knowledge of the technological aspects of the technique.

Key words: Anemia; Diagnosis; Hematological disorders; Hematopathology; Hemoglobin; Hemoglobinopathies; High-performance liquid chromatography; Laboratory instrumentation; Red blood cells; Thalassemia

Core tip: Interpretation of cation-exchange high-performance liquid chromatography requires in-depth knowledge of the biological basics of the disorders of hemoglobin with knowledge of the technological aspects of the technique. Pathologists may be unaware of the nuances of the technique, the rigorous quality control required and the approach to pitfalls that may be encountered. Here we list the most common of these, and based on literature and our experience, attempt to

guide novices in this exciting and useful technology.

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INTRODUCTION

Cation-exchange high-performance liquid chromatography (CE-HPLC) of a red blood cell lysate is now established as a rapid, accurate and reproducible diagnostic technique to separate various human hemoglobin fractions[1]. It is performed routinely in many laboratories, mostly on fully automated systems. Interpretation of HPLC chromatograms is a complex process requiring inputs from the clinical background of the case as well as complete blood count data[1,2]. Here, we briefly discuss a few technological aspects that influence results, and therefore are concern to reporting pathologists.

PRINCIPLE OF CE-HPLC

The basic principle of CE-HPLC involves passing the analyte of interest (a mixture of hemoglobins in solution) at a high pressure (approximately 100-200 kg/cm^2) through a cylindrical column packed with small spherical particles (typically 5 μm diameter silica gel, called the stationary phase). Very small sample volumes (usually approximately 5 μL) are applied to the column. Different hemoglobins adsorb onto the silica packing with different intensities based on their ionic interactions. The column is then perfused by a buffer (mobile phase) that constantly varies in pH and ionic strength. Different hemoglobins elute out with the perfusing buffer at different but characteristic time points in response to the continually changing salt gradient[3]. Another variable that affects elution/retention times, the column temperature, is kept fixed throughout the approximately 5.0 to 6.0 min run (Figure 1A).

HEMOGLOBIN DETECTION AND ASSIGNMENT OF WINDOWS

The eluted hemoglobin fractions are detected by a flow-cell type photometer that records changes in absorbance at 415 nm (hemoglobin) and 690 nm (background) on an integrating computer system. A chromatogram is generated displaying time on the X-axis and percentages on the Y-axis (Figure 1). The area under the absorption peak approximates the percentage of the fraction detected, and each fraction is assigned a window (*i.e.*, range of retention times). The software controls for overlapping/merging peaks by dropping vertical axes at the troughs[3,4]. The report prepared incorporates numerical as well as graphical data, and their analysis is discussed next. We use the very widely applied Bio-Rad Variant Ⅱ Turbo output (Bio-Rad Laboratories, Hercules, United States) using the β-Thal Short Programme for illustration (Figure 1), however, the principles remain similar even on other systems[5]. The interpretation of various chromatogram regions and peaks in various windows is summarized in Table 1. A specimen chromatogram with the peaks highlighted is shown in Figure 2.

CALIBRATORS AND CONTROLS

All HPLC runs are preceded by priming and then calibration of instrument. Separate calibration factors are obtained for HbA2 and HbF as ratios of expected to obtained values. Since the two values should ideally be equal (*i.e.*, a ratio of 1) these are deemed to have passed if they lie between 0.7 and 1.3. These calibration factors are then applied for all subsequent patient samples. The retention time of HbA2 in the calibrator is also a useful indicator of run reliability. Normally it lies between 2.60-2.70 min, and the instrument may need temperature adjustments if wider deviations occur. This is especially common as the column cartridge ages; usual cartridge lifetimes being around 250 injections. Bi-level controls, one normal (HbF 1%-2%, HbA2 1.8%-3.2%) and one elevated (HbF 5%-10%, HbA2 4%-6%), should be analyzed at the beginning as well as the end of each set of patient specimens. The high control in case of Bio-Rad instruments also contains a variant peak that must elute in the S-window. All peaks must be symmetrical, temperature variations being the most common cause again of asymmetry[3,6].

METHODOLOGICAL CONSIDERATIONS DURING REPORTING

Once the preliminary checks have passed, reporting of patient samples can proceed. During reporting, attention must be directed to the following areas.

Total area of analysis

This must lie between 1 to 3 million μVolt-seconds (Figure 1B). Specimens with lower areas (due to anemia) or increased values (due to polycythemia) must be re-analysed after appropriate manual concentration by removing plasma or dilution by removing red cells respectively[6].

Cases with total area > 100%

The total area is the sum of all individual peaks' areas and can therefore, in the presence of overlapping peaks, can cross 100%. This is especially common in patients with beta-thalassemia major, where the HbF peak overlaps and usually obliterates P2 and P3. In such

Table 1 Interpretation of various chromatogram regions and commonly encountered peaks in various high-performance liquid chromatography windows (retention time ranges obtained from Bio-Rad kit inserts)

Region/window	Retention time	Interpretation
Pre-integration region	< 1 min	Bilirubin, Hb H, Hb Barts, modified HbF
P1 peak	0.74	A minuscule peak usually found in specimens with increased HbF
F window	0.98-1.22	HbF, Hb Okayama
P2 window	1.28-1.50	Glycated HbA
P3 window	1.50-1.90	Aged samples, HbJ-Meerut, modified HbE
A window	1.90-3.10	HbA, Glycated HbS, intact Hb Koln
A2 window	3.30-3.90	HbE, Hb D-Iran, Hb Lepore, Hb G-Koushatta, Hb Zurich, Hb Korle Bu
D window	3.90-4.30	Hb D-Punjab, Hb G-Philadelphia
S window	4.30-4.70	HbS, Hb Q-Thailand, Hb Manitoba
C window	4.90-5.30	HbC, Hb Constant Spring, Hb Agenogi
Further unknown peaks	> 5.30	Hb Q-India

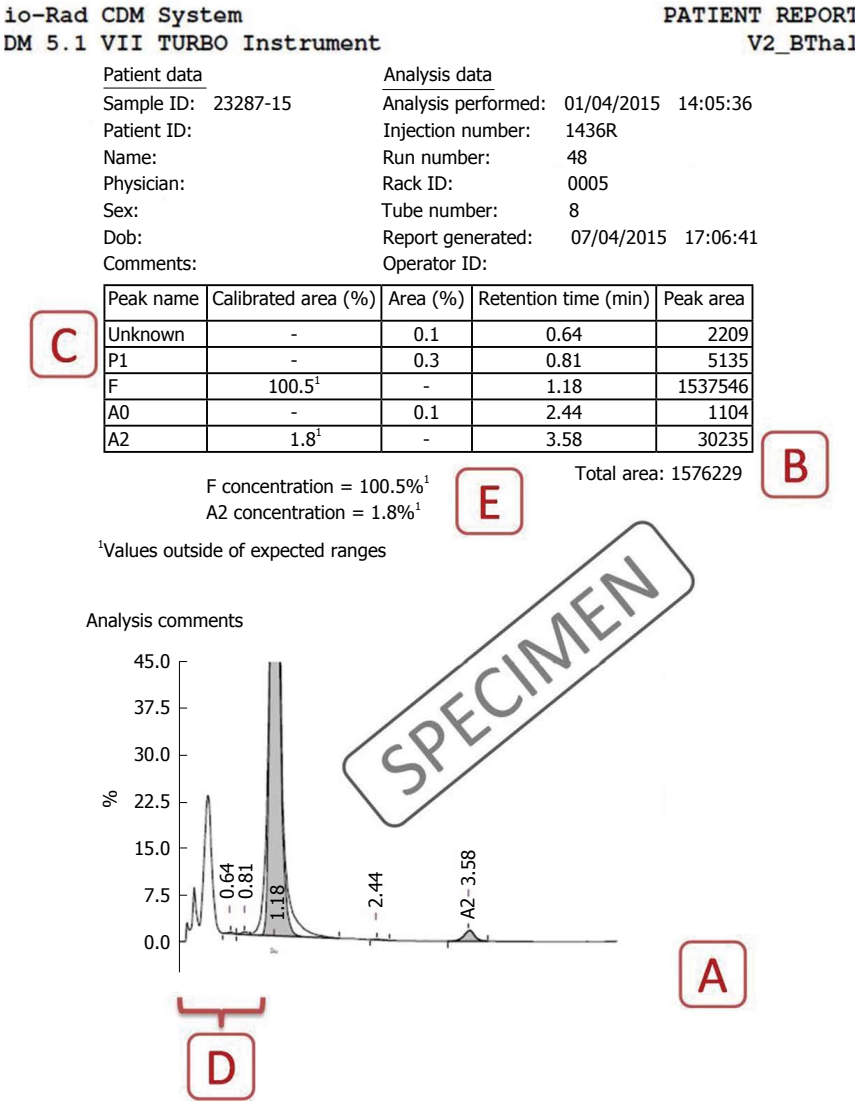

Figure 1 Specimen cation-exchange high-performance liquid chromatography output from the Bio-Rad Variant Ⅱ Turbo instrument (Bio-Rad Laboratories, Hercules, United States) using the β-Thal short programme. Label A indicates the total time of analysis (X-axis) is approximately 5 to 6 min; Label B indicates that the total area of analysis should lie between 1 and 3 million; Label C shows the unknown peaks that may occur, especially in the P2 and P3 regions; Label D depicts the preintegration phase (< 1 min) is not reflected in the table above and should be analyzed on the chromatogram; Label E shows where the problem of HbF concentration being calculated as > 100% is present in this case. Please see text for details of resolution of this problem.

Peak name	Area (%)	Retention time (min)	Peak area	Old CF	New CF
P1	-	0.74	655	-	-
F	6.3	1.10	170743	1.004	1.025
P2	-	1.30	102589	-	-
P3	-	1.71	123335	-	-
A0	-	2.35	2090989	-	-
A2	7.5	3.60	203063	0.887	0.901

Total area: 2691374

F concentration = 6.5%

A2 concentration = 6.8%

Analysis comments

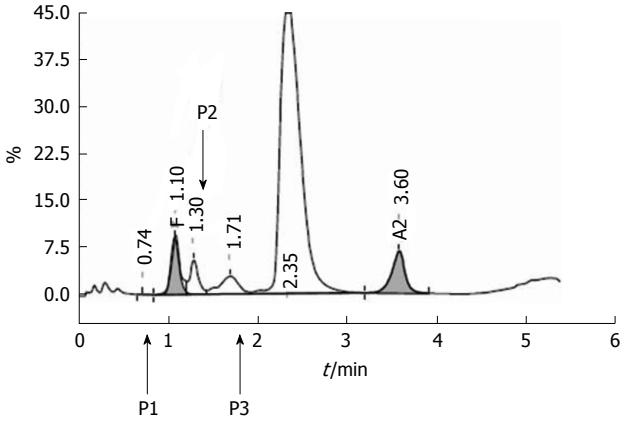

Figure 2 A specimen chromatogram to show the various peaks that may occur in health and disease. The patient, with elevated HbA2 and mildly elevated HbF, most likely had β-thalassemia trait with increased HbF (HbF levels between 1%-5% are found in approximately 30% of β-thalassemia trait cases, and occasional ones may have even higher levels). Calibrator data had shown above.

TECH ID# 2
VIAL# 20
Sample ID# 00000000000000001152

Analyte	ID	%	Time	Area
P2		92.7	1.24	1405107
Unknown	1	0.2	2.16	2374
A0		3.0	2.62	45723
A2		4.3	3.69	59862
		Total area		1513066

F 0.0% A2 4.3%

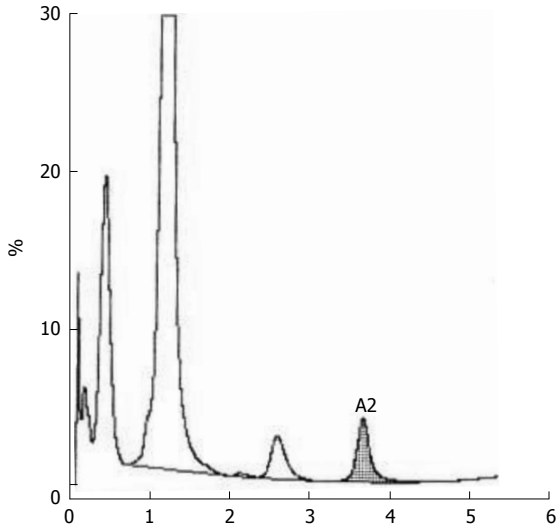

Figure 3 A case of thalassemia major with a very prominent and broad-based HbF being reported by the instrument (an older Bio-Rad Variant) as a P2-peak.

situations, the percentages can be calculated manually, by taking the area of the peak of interest, and dividing it by total area to get the proportion. For example, in Figure 2, the HbF%, instead of the implausible 100.5% can be calculated as F-peak area × 100 ÷ total area (*i.e.*, 1537546 × 100 ÷ 1576229 = 97.5%).

Small unknown peaks

One or more unknown peaks often occur around the P2/P3 window (retention times 1.3-1.8; Figure 1C). These may be safely ignored if ⩽ 1% of total area[6]. Unknown peaks at longer retention times and those > 1% should be paid greater attention as transfusion-transmitted peaks, HbA2' may present as small peaks[7]. One may also review the sample run previously as carryover peaks are also usually small.

Misidentified hemoglobin fractions

In rare cases with very large abnormal peaks, the entire fraction may be misassigned to either another category, or as an unknown peak. This was commoner in older generation analyzers (like the Variant), but can still occur with broad-based HbD and HbF peaks (Figure 2). Alternative techniques are then required to establish the identity of the unknown peak.

Pre-integration peaks

These peaks, with retention time < 1 min, are not reflected in the tabular data and need to be looked for on the chromatogram (Figure 1D). The causes of such peaks include HbH, Hb Barts, bilirubin and acetylated HbF (Figure 3). The clinical background and other HPLC findings usually indicate their nature, if found. In addition, bilirubin peaks are usually early, very sharp and thin. HbH peaks are usually dual and of low to moderate height, while post-translationally modified F are usually multiple with their height proportionate to the HbF%[1,3,4]. If required, the software settings may be readjusted manually to include such peaks. This may be especially useful in cases with HbH disease.

P2 and P3 peaks

These represent post-translationally modified adult hemoglobin (HbA0) and show normal ranges of 3.8 ± 0.7 and 4.3 ± 0.4 respectively (unpublished data). P2 is comprised of glycated hemoglobin and levels ⩾ 6.5% should be mentioned in the report with the suggestion to exclude diabetes mellitus. Low P2 levels are seen in cases with reticulocytosis. Elevated P3 may indicate HbJ-Meerut (an α-globin chain variant). It is also elevated in cases with the HbE variant (that elutes in the HbA2 window) and in aged specimens[1,3,4]. Incidentally, the P1-peak is virtually always absent in normal specimens.

In conclusion, although CE-HPLC is a rapid, convenient and reliable investigation for hemoglobin disorders, it involves several methodological issues and nuances. Reporting pathologists must be aware of these to extract maximum information from this technology.

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REVIEW

Clinical development of reovirus for cancer therapy: An oncolytic virus with immune-mediated antitumor activity

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Abstract

Reovirus is a double-stranded RNA virus with demonstrated oncolysis or preferential replication in cancer cells. The oncolytic properties of reovirus appear to be dependent, in part, on activated Ras signaling. In addition, *Ras*-transformation promotes reovirus oncolysis by affecting several steps of the viral life cycle. Reovirus-mediated immune responses can present barriers to tumor targeting, serve protective functions against reovirus systemic toxicity, and contribute to therapeutic efficacy through antitumor immune-mediated effects *via* innate and adaptive responses. Preclinical studies have demonstrated the broad anticancer activity of wild-type, unmodified type 3 Dearing strain reovirus (Reolysin®) across a spectrum of malignancies. The development of reovirus as an anticancer agent and available clinical data reported from 22 clinical trials will be reviewed.

Key words: Reovirus; Type 3 Dearing; Oncolytic virus; Ras; Epidermal growth factor receptor; Clinical trial; Preclinical; Immune modulation

Core tip: Reovirus has demonstrated oncolysis or preferential replication in cancer cells. The anticancer activity of reovirus has been demonstrated across a spectrum of malignancies in the preclinical setting. The relatively tolerable toxicity profile of reovirus renders it an attractive agent as part of combination therapy in cancer treatment. Reovirus-mediated immune modulation contributes to its antitumor activity *via* innate and adaptive immune responses and renders it an attractive component of immunotherapy. Here we compile the most extensive list of clinical trials investigating the anticancer efficacy of reovirus to date.

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INTRODUCTION

Reovirus and mechanism of oncolysis

The *Reoviridae* family of viruses consists of six genera, three of which including rotavirus, orbivirus, and reovirus are known to infect animals and humans, while the other three are known to infect plants and insects[1,2]. In 1959, the name reovirus was given to a virus commonly isolated from the respiratory and enteric tract that seldom caused few, if any, clinical symptoms (orphan virus)[3]. However, when symptomatic, reovirus infection is characterized by mild enteric and respiratory symptoms in humans[1-5]. Wild-type reovirus is ubiquitous throughout the environment with seropositivity having been documented in as many as 70%-100% of subjects[3]. There exists several serotypes of reovirus [type 1 Lang, type 2 Jones, type 3 Abney, and type 3 Dearing (T3D)] that have been identified by antibody hemagglutination-inhibition and neutralization studies[2,3,5].

Reovirus is approximately 80 nm in diameter and comprised of a protein shell with outer and inner components that altogether create an icosahedral capsid housing ten segments of double-stranded RNA (dsRNA)[1,2,4-7]. It has been more than 30 years since wild-type reovirus was demonstrated to replicate preferentially in transformed cell lines but not in normal cells[8,9]. The means by which reovirus oncolysis occurred remained elusive until rodent cell lines transformed with genes encoding the epidermal growth factor receptor (EGFR) and a truncated form of the EGFR, possessing constitutive tyrosine kinase activity but lacking the extracellular ligand-binding domain, demonstrated increased susceptibility to reovirus infection and thereby proposing that EGFR-mediated pathways facilitated reovirus infection[10,11]. Indeed, transfection with constitutively activated *Ras* oncogenes or son of sevenless in NIH-3T3 fibroblasts resulted in increased vulnerability to reovirus infection and elucidated the involvement of activated Ras signaling pathways in reovirus oncolysis[12,13].

Given that approximately 30% of all cancers in humans have been linked to activating *Ras* mutations, subsequent studies investigated prospective downstream mediators of Ras that may be critical to reovirus oncolysis and implicated, in particular, the Ras/RalGEF/p38 pathway in promoting preferential reovirus replication[14,15]. Additionally, it was determined that dsRNA-activated protein kinase (PKR), which is normally activated in the presence of viral transcripts and inactivates eukaryotic initiation factor 2α (eIF-2α), protein synthesis, and viral replication, is kept inactivated in *Ras*-transformed cells thereby providing the link between PKR and an activated Ras signaling pathway in reovirus oncolysis[13,16]. Aside from viral translation, *Ras*-transformation has been shown to promote oncolysis by affecting other steps of the reovirus infectious life cycle including viral disassembly or uncoating, production of viral progeny with boosted infectivity, progeny release through increased apoptosis, and spread of virus in later cycles of infection[17-19].

PRECLINICAL DEVELOPMENT OF REOVIRUS

Monotherapy

Given the wide-reaching implications of activated *Ras* mutations in human cancers, the first proof-of-concept preclinical studies involved tumors established from v-erbB-transformed murine NIH-3T3 fibroblasts and human U87 glioblastoma cells implanted in severe combined immune deficient (SCID) mice that demonstrated marked tumor regression in approximately 80% of mice following single intratumoral injections of reovirus by day 12 and week 4, respectively[20]. However, SCID mice represented a non-ideal model for reovirus antitumor studies given that approximately 50%-60% of reovirus-treated animals experienced limb necrosis and death[20]. The "Black Foot" syndrome has been characterized by infection with live reovirus of venule endothelial cells and myocardial and musculoskeletal myocytes leading to vasculitis, localized hemorrhage, and/or thrombosis in the extremities of SCID mice[21]. Activated Ras signaling pathways are present in a majority of malignant gliomas, and accordingly, reovirus demonstrated antitumor activity in 83% of malignant glioma cells *in vitro*, in 2 subcutaneous and 2 intracerebral human malignant glioma models *in vivo*, and in 100% of glioma specimens *ex vivo*[22]. In medulloblastoma cell lines, reovirus translation was restricted to cell lines with higher levels of activated Ras, and intratumoral injections of reovirus prolonged survival in orthotopic *in vivo* animal models of medulloblastoma with spinal and leptomeningeal metastases[23].

The incidence of activated *Ras* mutations in colon cancer is approximately 50%[15]. The significance of *Ras* transformation in reovirus oncolysis of colon cancer cells has also been highlighted in K-*Ras* knockdown murine colorectal cancer cells that demonstrated complete nullification of reovirus-induced apoptosis compared to control[24]. Indeed, treatment with reovirus exhibited significant antitumor effects in human colorectal cancer *in vitro* and *in vivo* characterized by elevated Ras activity in colon cancer cell lines and restriction of reovirus infection to tumor cells when compared to controls[25]. Other studies also demonstrated the antitumor efficacy of reovirus *in vitro* in colon cancer cell lines, *in vivo* in rodent models of colorectal liver metastases, and notably, in fresh human colorectal tissue isolates that required the processing of virions to infectious subvirion particles (ISVPs) and proper localization and quantity of junctional adhesion molecule-1 on tumor cells for productive lysis[26-28]. Furthermore, colon cancer cell lines HEK293 and HCT116 demonstrated sensitization to reovirus-induced apoptosis by downregulation of nuclear

factor-kappa B (NF-κB) through inhibition of glycogen synthase kinase-3β[29].

In adenocarcinomas of the pancreas, the incidence of K-*Ras* mutations is among the highest in human cancer (approximately 90%)[15]. Not surprisingly, reovirus demonstrated potent cytotoxicity in 100% of pancreatic cancer cell lines *in vitro* and induced regression in 100% of subcutaneous tumor mouse models *in vivo*[30]. Interestingly, antitumor activity was seen in BxPC3 pancreatic cancer cells, which are known to have normal K-*Ras* oncogenes, treated with reovirus *in vitro* and *in vivo* though the reovirus-induced cytotoxicity observed in these cells was attributed to overall increased Ras activity, a concept reintroduced below[30]. Administration of reovirus also induced regression in immunocompetent hamster models of pancreatic cancer with liver and peritoneal metastases compared to controls[31,32].

Although the incidence of H-*Ras* mutations has been reported as high as 17% in cases of bladder carcinoma, activated EGFR-mediated pathways are present in up to 50% of cases of transitional cell carcinoma (TCC) of the bladder[15,33]. Treatment of co-cultured spheroids established by culturing TCC of the bladder cell lines and fibroblasts with reovirus demonstrated selective killing of tumor cells by lysis or induction of apoptosis *in vitro*[33]. Additionally, intravesical administration of reovirus resulted in significantly higher tumor-free survival in an orthotopic rat model of bladder cancer compared to control[34]. Along similar lines of thought, the incidence of N-*Ras* mutations in melanoma is relatively lower (approximately 8%-19%) compared to those found in colon and pancreatic cancers[15]. Nevertheless, human melanoma cell lines and murine xenograft models of melanoma were susceptible to tumor killing by reovirus with implications towards the role of the immune system in reovirus oncolysis (which will be further discussed later)[35].

Interestingly, activating *Ras* mutations in breast cancer are relatively rare though unregulated stimulation of Ras signaling pathways through mediators such as human EGFR 2 (Her-2 or ErbB-2) and its homologue Neu, both tyrosine kinases of the EGFR family, and the Src family of nonreceptor tyrosine kinases can occur highlighting the concept that activated Ras signaling rather than mutations in the Ras protein itself can be important to disease pathogenesis[3-5,36]. Accordingly, reovirus demonstrated significant antitumor effects *in vitro* in breast cancer cell lines characterized by resistance to infection in normal cell lines, *in vitro* in breast cancer stem cells, and *in vivo* in animal tumor models including models of brain and leptomeningeal metastases[36-39]. Furthermore, the presence of replicating reovirus was confirmed in *ex vivo* surgical breast cancer specimens[36]. Notably, there was no observed relationship between susceptibility to reovirus infection and HER2 expression, *in vitro*, though levels of Ras activity were higher in breast cancer cell lines when compared to control[37]. Ovarian cancer represents another example in which activating *Ras* mutations are rare but increased Ras signaling *via* increased activation of Her-2/Neu and/or Src likely contribute to pathogenesis[4,5,25]. Treatment with reovirus resulted in potent antitumor activity, when compared to controls, in ovarian cancer cell lines *in vitro* highlighted by increased reovirus protein synthesis in tumor cell lines but not in normal cells, in a human ovarian SKOV3 cell line implanted in the flanks of mice *in vivo*, and in a murine ascites model of human ovarian cancer highlighted by prolonged survival in those treated with intraperitoneal injections of live virus every 2 wk[25]. All 3 *ex vivo* human ovarian tumor surgical biopsy specimens also demonstrated susceptibility to reovirus infection[25].

Similarly, marked cytopathic effects and inhibition of tumor growth were observed with reovirus treatment, *in vitro* and *in vivo*, in cancers where relatively little has been known, historically, about the involvement of *Ras* mutations in transformation such as head and neck cancer, prostate cancer, and sarcomas[40-42]. Intriguingly, although reovirus-induced cytotoxicity was observed in several head and neck carcinoma cell lines, correlative analyses revealed no associations between phosphorylated eIF-2α or EGFR levels and cytopathic effects suggesting that reovirus oncolysis appears to occur independently of PKR, Ras signaling, and EGFR signaling pathways[43,44].

Hematologic malignancies posed a perplexing dilemma regarding their susceptibility to reovirus infection given the near absence of N-*Ras* mutations particularly in chronic lymphocytic leukemia (CLL) and non-Hodgkin lymphomas (NHLs) such as follicular lymphoma (FL) and diffuse large B-cell lymphoma (DLBCL)[15,45]. Nevertheless, it was hypothesized that certain hematologic malignancies may still be amenable to reovirus therapy from knowledge that the break point cluster-Abelson (Bcr-Abl) nonreceptor tyrosine kinase present in 95% of chronic myelogenous leukemia is dependent on *Ras* activation, *Myc* oncogenes coordinate with *Ras* in B-cell transformation, specific ligand-receptor interactions in CLL lymphoid cells stimulate Ras signaling, and mutations in a proto-oncogene member of the *Ras* superfamily is present in up to 46% of DLBCLs[3,45]. Indeed, reovirus treatment of human lymphoma cells produced antitumor effects in all 4 DLBCL cell lines and 2 out of 5 Burkitt lymphoma cell lines *in vitro* highlighted by increased reovirus protein synthesis and progeny production in sensitive cell lines compared to resistant cell lines and *in vivo* in a Burkitt cell line sensitive to reovirus implanted in mice but not in a xenograft model of a previously determined resistant Burkitt cell line[45]. Furthermore, all *ex vivo* human primary CLL samples and a majority of NHL samples including Burkitt lymphoma, mantle cell lymphoma, and DLBCL were susceptible to reovirus oncolysis while a majority of FL specimens were resistant[45].

Treatment of acute myeloid leukemia (AML) with reovirus showed marked antitumor responses in 2 out 4 AML cell lines *in vitro* and in 8 out of 10 peripheral blood primary AML specimens *ex vivo*[46]. Concordant

with prior findings, a FL cell line was resistant to reovirus therapy *in vitro* and *in vivo* while mantle cell lymphoma cell lines displayed a heterogenous response to reovirus that correlated with levels of activated Ras and proteolytic disassembly of reovirus into ISVPs *in vitro*[47]. The discrepancies in sensitivity to reovirus infection between various hematologic malignancies have been attributed, in part, to differential *Ras* activation and interferon sensitivities[4,5,45,47]. Reovirus induced cell death *via* apoptotic and autophagic pathways in a majority of multiple myeloma cell lines *in vitro* with sensitivity conferred to *ex vivo* tumor specimens as well[48]. Reovirus also showed meaningful inhibition of tumor growth in *in vivo* multiple myeloma models compared to control, and treatment with reovirus did not abrogate human stem cell repopulation and differentiation *in vivo*[48]. Earlier studies revealed that reovirus did not affect hematopoietic progenitor stem cells, and the mixture of reovirus with human monocytic and myeloma cancer cell lines *in vitro* and *ex vivo* tumor cells of DLBCL, CLL, Waldenström macroglobulinemia, and small lymphocytic lymphoma showed complete purging of disease in patient products of apheresis[49]. The use of reovirus as a purging strategy for autologous stem cell transplantations has since been an emerging concept with demonstrated efficacy in breast cancer and multiple myeloma[50,51].

Combination therapy

The earliest preclinical studies involving reovirus in combination therapy entailed L1210 murine leukemia cells and EL4 murine lymphoma cells treated with the chemotherapeutic agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) followed by treatment with reovirus that increased survival in ascites tumor mouse models when compared to controls and were among the first to illustrate that resistance of surviving animals to challenges with homologous tumor was orchestrated by an immune-mediated process[52-54]. Reovirus in combination with radiation therapy, when compared to controls, produced enhanced apoptosis across head and neck, colorectal, and breast cancer cell lines *in vitro* (independent of treatment sequence or schedule and without affecting viral replication at clinically relevant radiation doses) and delayed tumor growth in colorectal cancer and melanoma models *in vivo*[55]. Criteria for therapeutic enhancement were met for ewing sarcoma (ES) and osteosarcoma murine xenografts and rhabdomyosarcoma and ES murine xenografts treated with reovirus in combination with cisplatin and reovirus in combination with radiotherapy [4 gray (Gy) daily × 5 fractions], respectively[40].

In murine melanoma xenografts, metronomic dosing of high-dose cyclophosphamide with reovirus permitted access to tumors by therapeutically high levels of virus while reducing serious toxicities associated with ablation of neutralizing antibody titers, and cisplatin with reovirus significantly inhibited tumor growth compared to controls without affecting neutralizing antibody response though cisplatin reduced the inflammatory cytokine response to reovirus[56,57]. Treatment with reovirus and cyclosporin A significantly inhibited tumor growth in a murine *Ras*-transformed fibroblastic xenograft while reovirus with cyclosporin A or T-cell depletion significantly improved survival in a murine metastatic lung cancer model compared to controls[58]. Although reovirus alone demonstrated potent cytotoxicity in 7 of 9 non-small cell lung cancer (NSCLC) cell lines *in vitro*, heterogenous synergistic effects on cell killing were observed with reovirus in combination with cisplatin, gemcitabine, or vinblastine on NSCLC cancer cell lines *in vitro*[59]. The reovirus and paclitaxel combination, however, showed synergistic cell killing in all NSCLC cell lines *in vitro* characterized by enhanced apoptosis[59].

More recently, although trastuzumab and reovirus monotherapy both inhibited tumor growth *in vitro*, treatment with reovirus was found to sensitize gastric cancer cells that overexpressed HER2 to apoptosis when combined with trastuzumab[60]. However, in HER2 low expressing cells, reovirus monotherapy or in combination with trastuzumab increased apoptosis *in vitro*, but there was no reduction in growth when treated with trastuzumab alone[60]. Further analysis showed that reovirus induced expression of TRAIL, a protein implicated in promoting apoptosis, without upregulating TRAIL receptors. TRAIL expression was increased with both trastuzumab and reovirus therapy, but this effect was enhanced by combination therapy[60].

Similar synergistic antitumor effects have been established, when compared to controls, in combination regimens involving: (1) reovirus with cisplatin and paclitaxel in head and neck cancer *in vitro* characterized by enhanced apoptosis and cell cycle disruption (though without enhancing reovirus replication) and *in vivo*; (2) reovirus with bortezomib in pancreatic cancer *in vitro* and *in vivo* characterized by enhanced levels of ER stress and apoptosis; (3) reovirus with cyclosporin A in a murine model of colorectal liver metastases; (4) reovirus and gemcitabine in human colon cancer *in vitro* and *in vivo*; and (5) reovirus with paclitaxel, vincristine, cisplatin, doxorubicin, or docetaxel in prostate cancer *in vitro* highlighted by the greatest synergism in the reovirus and docetaxel combination with enhanced apoptosis and microtubule stabilization[27,61-64]. Reovirus and docetaxel also produced significant tumor growth retardation in a murine prostate cancer xenograft[63]. Interestingly, reovirus in combination with Newcastle disease virus or parvovirus resulted in significant synergistic antitumor responses in glioblastoma cell lines *in vitro* with an efficient rate of co-infection and without affecting the kinetics of viral replication among the viruses[65]. Furthermore, reovirus with Newcastle disease virus significantly inhibited tumor growth in a murine glioblastoma xenograft compared to control without significant toxicity though the experiments were terminated 12 d after virus injection[65].

In sum, preclinical studies have demonstrated the broad anticancer activity of reovirus across a spectrum

of malignancies including colon, breast, ovarian, lung, skin (melanoma), neurological, hematological, prostate, bladder, and head and neck cancer which have ultimately provided the basis for human clinical trials[1,5,6,66]. The three serotypes of reovirus including type 1 Lang, type 2 Jones, type 3 Abney, and T3D all have demonstrated oncolytic properties, but the T3D strain has been most extensively studied as an anticancer agent and is the only therapeutic wild-type reovirus in clinical development under its proprietary formulation, Reolysin®, developed by Oncolytics Biotech Inc. (Calgary, Canada)[2-4,67]. Thus far, there are a total of 34 clinical trials involving reovirus in the treatment of a variety of cancers that are both completed and ongoing (http://www.oncolyticsbiotech.com/clinical-trials). Clinical data available and reported from 22 clinical trials will now be discussed (Tables 1 and 2).

CLINICAL DEVELOPMENT OF REOVIRUS

Phase Ⅰ trials

The first phase Ⅰ trial (REO 001) involved administration of intralesional reovirus in patients with advanced solid tumors and histologically confirmed cutaneous lesions[68]. In a dose-escalation design, doses of 1×10^7 plaque forming units (PFU) once weekly up to maximum doses of 1×10^{10} PFU once weekly were used[68]. Out of 19 patients, dose-limiting toxicities (DLTs) and a maximum-tolerated dose (MTD) were not observed even at maximum dose[68]. The most common treatment-related adverse events (AEs) included nausea (79%), vomiting (58%), and local erythema of injection site (42%) while fevers/chills and transient flu-like symptoms accounted for 37% and 32%, respectively[68]. The best overall response ≥ 6 wk was complete response (CR) in 1 (5.3%), partial response (PR) in 2 (10.5%), stable disease (SD) in (21.1%)[68].

REO 002 enrolled 6 patients with localized prostate cancer who received a single intratumoral injection of 1×10^7 PFU of reovirus 3 wk prior to planned prostatectomy as definitive cancer treatment[42]. There were no DLTs or grade 3 or higher toxicities observed and the most common AE included mild flu-like illness in 4 out of 6 patients[42]. In all patients, prostate-specific antigen (PSA) levels did not significantly fluctuate from baseline, and pathologic specimens showed moderate to strong staining for reovirus proteins localized to areas of cancer but sparing of adjacent benign areas and remote areas of cancer in 5 patients[42].

Another phase Ⅰ study (REO 003) involved single stereotactic intralesional injection of reovirus at doses ranging from 1×10^7 tissue culture infectious dose-50 ($TCID_{50}$) to 1×10^9 $TCID_{50}$ in 12 patients with progressive or recurrent malignant gliomas[69]. The MTD was not reached even at maximum doses and there were no DLTs observed with the only grade 3 or higher treatment-related AE being an elevation in γ-glutamyl transpeptidase[69]. The median time to disease progression (TTP) was 4.3 wk (range 2.6-39 wk), median overall survival (OS) was 21 wk (range 6-234 wk), and best overall response was SD in 1 patient with TTP of 39 wk[69]. REO 007, a multicenter phase Ⅰ study, aimed to determine DLTs, MTD, and target lesion response rate after administering reovirus *via* intratumoral infusion in 15 patients with recurrent malignant gliomas[70]. Similarly to REO 003, the MTD was not achieved at maximum doses. Only three patients suffered from convulsions, a grade 3 AE, which does occur commonly in patients with intracranial tumors[70]. Additionally, only one of these three grade 3 AEs was possibly related to infusion of reovirus[70]. During the study period of 24 wk, ten patients were reported to have stable disease, four with progressive disease, and one with partial response[70]. However, ultimately 12 out of the 15 patients did have progressive disease with the median time to progression being 61 d (range 29-150) and the median survival being 140 d (range 97-989)[70]. The one patient that did achieve a partial response did receive the maximum dose[70].

REO 004 included 18 patients with advanced solid tumors treated with intravenous (*IV*) reovirus from 1×10^8 $TCID_{50}$ to 3×10^{10} $TCID_{50}$ once every 28 d in which the latter dose was declared the MTD due to protocol termination once the protocol-defined highest dose was reached[71]. No DLTs were observed and the most common AEs included myalgia, fatigue, and fever[71]. Out of 18 patients, the best overall response was PR > 5 cycles in 1 patient (5.6%) with taxane and anthracycline refractory breast cancer (whose post-treatment chest wall biopsy showed viral replication and extensive necrosis consistent with reovirus activity) and SD > 1 cycle in 7 patients (38.9%) for a clinical benefit rate (CBR) of about 45% (combined CR, PR, and SD)[71]. Of note, 5 patients had *Ras* mutations and 1 patient had a *Braf* mutation, and the formation of neutralizing anti-reovirus antibodies (NARAs) bore no relationship to clinical benefit while those with detectable viral shedding appeared to have greater benefit[71]. One phase Ⅰ study (REO 005) pitted *IV* reovirus against various refractory or metastatic cancers, and a MTD was reached at a dose of 3×10^{10} $TCID_{50}$ once daily for 5 d every 28 d by virtue of being the highest dose available for administration (this subsequently also became the recommended phase Ⅱ dose)[72]. No DLTs were observed and the most common AEs were fever, fatigue, and headache[72]. Out of 33 enrolled patients, the best overall response was SD > 7 wk in 10 patients, and no relationships between SD to dose or duration of reovirus therapy were established[72].

REO 006 enrolled 25 patients with various refractory or progressive solid cancers in a two-stage dose-escalation design where phase Ia treated patients with 1×10^8 $TCID_{50}$ to 1×10^{10} $TCID_{50}$ intratumoral injection of reovirus on days 2 and 4 with 20 Gy local irradiation daily × 5 fractions while phase 1b treated patients with 1×10^{10} $TCID_{50}$ intratumoral injection of reovirus twice weekly for 1-3 wk with 36 Gy local irradiation × 12 fractions over 16 d[73]. There were no DLTs observed, a MTD was not reached, and the most common treatment-related AEs

Table 1 Phase Ⅰ trials involving reovirus

Phase	Malignancy	Dosing regimen	Clinical response
Ⅰ (REO 001)	Various advanced or refractory solid malignancies	1×10^7 PFU to 1×10^{10} PFU intralesional injection once or 3 × weekly (dose escalation)	Out of 19 patients, best overall response ≥ 6 wk was CR in 1 with Klatskin (5.3%), PR in 2 with head and neck cancer (10.5%), SD in 4 1 with head and neck, 1 with melanoma, 1 with breast cancer, 1 with Kaposi's (21.1%)
Ⅰ / translational (REO 002)	Localized prostate cancer	1×10^7 PFU single intratumoral injection 3 wk prior to planned prostatectomy	Out of 6 patients, all did not exhibit significant fluctuations in PSA from baseline. Five of 6 patients showed staining for reovirus proteins localized to cancer areas but sparing of adjacent benign and remote cancer areas. Pathologic specimens showed peritumoral inflammation in 4 patients, apoptosis in 4 patients, and necrosis in 2 patients
Ⅰ (REO 003)	Advanced or recurrent malignant gliomas	1×10^7 $TCID_{50}$ to 1×10^9 $TCID_{50}$ single stereotactic intralesional injection (dose escalation)	Out of 12 patients, best overall response was SD in 1 patient with oligo-astrocytoma with a TTP of 39 wk. The overall median TTP was 4.3 wk (range 2.6-39 wk), and median OS was 21 wk (range 6-234 wk)
Ⅰ (REO 004)	Various advanced or refractory solid malignancies	60-min *IV* infusion from 1×10^8 $TCID_{50}$ to 3×10^{10} $TCID_{50}$ once every 28 d (dose escalation)	Out of 18 patients, best overall response was PR > 5 cycles in 1 patient with breast cancer (5.6%) and SD > 1 cycle in 7 (5 with ovarian cancer, 1 with carcinoid, 1 with STS, 38.9%); CBR of about 45%
Ⅰ (REO 005)	Various advanced or refractory solid malignancies	60-min *IV* infusion from 1×10^8 $TCID_{50}$ once every 28 d to 3×10^{10} $TCID_{50}$ once daily for 5 d every 28 d (dose escalation); *IV* reovirus 3×10^{10} $TCID_{50}$ once daily for 5 d every 28 d became recommended phase Ⅱ dose	Out of 33 enrolled patients, best overall response was SD > 7 wk in 10 patients (2 with colon cancer, 2 with prostate cancer, 2 with STS, 1 with lung cancer, 1 with TCC of the bladder, 1 with melanoma, 1 with endometrial cancer)
Ⅰ (REO 006)	Various advanced or refractory solid malignancies	1×10^8 $TCID_{50}$ to 1×10^{10} $TCID_{50}$ intratumoral injection on days 2 and 4 with 20 Gy local irradiation daily × 5 fractions *vs* phase 1b: 1×10^{10} $TCID_{50}$ intratumoral injection twice weekly from 1-3 wk with 36 Gy local irradiation × 12 fractions over 16 d (two-stage dose escalation); intratumoral 3×10^{10} $TCID_{50}$ × 2 injections with 20 Gy × 5 fractions and intratumoral 1×10^{10} TCID50 × 6 injections with 36 Gy × 12 fractions became recommended phase Ⅱ doses for short and prolonged palliative regimens, respectively	Out of 7 patients in phase 1a, best overall response was PR in 2 (esophageal adenocarcinoma and SCC of skin), SD in 5 (melanoma, pancreatic adenocarcinoma, SCC of larynx, and 2 with SCC of skin); out of 7 patients in phase 1b, 5 had PR (lung adenocarcinoma, colorectal cancer, ovarian adenocarcinoma, 2 with melanoma) and 2 had SD (melanoma) up to 3 mo post-treatment
Ⅰ (REO 007)	Recurrent malignant gliomas	72-h intratumoral infusion from 1×10^8 $TCID_{50}$ to 1×10^{10} $TCID_{50}$ (dose escalation)	Out of 15 patients enrolled, best overall response was SD in 10 patients during the study period of 24 wk. The median TTP was 61 d (range 29-150 d), and median survival was 140 d (range 97-989)
Ⅰ (REO 009)	Various advanced or refractory solid malignancies	60-min *IV* infusion from 1×10^9 $TCID_{50}$ to 3×10^{10} $TCID_{50}$ on day 1 (dose escalation) with 30-min *IV* infusion of gemcitabine 1000 mg/m^2 days 1 and 8 every 21 d (1×10^{10} $TCID_{50}$ reovirus on day 1 became recommended phase Ⅱ dose with gemcitabine)	Out of 10 patients, best overall response was PR after 4 cycles in 2 patients (1 with nasopharyngeal carcinoma, 1 with breast cancer) and SD for 4-8 cycles in 5 patients (median SD 72 d, range 36-112 d); CBR of 80%
Ⅰ (REO 010)	Various advanced or refractory solid malignancies	60-min *IV* infusion from 3×10^9 $TCID_{50}$ to 3×10^{10} $TCID_{50}$ days 1-5 (dose escalation) with 60-min *IV* infusion of docetaxel 75 mg/m^2 day 1 every 21 d (3×10^{10} $TCID_{50}$ reovirus days 1-5 every 21 d became recommended phase Ⅱ dose with docetaxel)	Out of 16 patients, best overall response was PR ≥ 2 cycles in 4 patients (1 with breast cancer who experienced CR in liver lesion, 1 with gastric cancer, 1 with gastroesophageal cancer, 1 with ocular melanoma) and SD ≥ 2 cycles in 10 patients (cancers included prostate, mesothelioma, SCC of head and neck, unknown primary, melanoma, esophageal cancer, pancreatic cancer); CBR of 88%
Ⅰ / translational (REO 013)	Colorectal cancer metastatic to the liver	60-min *IV* infusion of 1×10^{10} $TCID_{50}$ daily × 5 d between 6-28 d prior to planned radical resection of liver metastases	Out of 10 patients, 9 patients with resected tumor specimens demonstrated positive staining for reovirus that was greatest in tumor metastases compared to surrounding tumor stroma or adjacent normal liver. In addition, tissue analysis in 4 patients showed findings consistent with reovirus-associated apoptosis
Ⅰ (REO 022)	Metastatic colorectal cancer	60-min *IV* infusion from 1×10^{10} $TCID_{50}$ to 3×10^{10} $TCID_{50}$ days 1-5 every 28 d (dose escalation) with standard FOLFIRI doses (recommended phase Ⅱ dose was irinotecan 150 mg/m^2 with 3×10^{10} $TCID_{50}$ *IV* reovirus days 1-5 every 28 d)	Out of 18 patients, best overall response was PR in 1 patient (5%) and SD in 9 (50%) with median PFS in FOLFIRI-naïve patients of 7.4 mo (95%CI: 1.9-12.9 mo) and overall median PFS of 7.4 mo (95%CI: 0.6-14.1 mo)
Ⅰ (OSU-11148, NCI trial)	Refractory or relapsed multiple myeloma	60-min *IV* infusion from 3×10^9 $TCID_{50}$ to 3×10^{10} $TCID_{50}$ days 1-5 every 28 d (dose escalation)	Out of 12 patients, best overall response was SD with longest duration being 8 cycles. During cycle 1, 5 patients had decreased myeloma proteins, 3 had minimal increases, and 4 had progressive disease

PFU: Plaque forming units; CR: Complete response; PR: Partial response; SD: Stable disease; PSA: Prostate-specific antigen; $TCID_{50}$: Tissue culture infectious dose-50; TTP: Time to disease progression; OS: Overall survival; *IV*: Intravenous; STS: Soft tissue sarcoma; CBR: Clinical benefit rate; TCC: Transitional cell carcinoma; Gy: Gray; SCC: Squamous cell carcinoma; FOLFIRI: Irinotecan/fluorouracil/leucovorin; PFS: Progression-free survival.

Table 2 Phase Ⅰ/Ⅱ, Ⅱ, and Ⅲ trials involving reovirus

Phase	Malignancy	Dosing regimen	Clinical response
Ⅰ/Ⅱ (REO 011)	Various advanced or refractory solid malignancies	60-min *IV* infusion from 3×10^9 $TCID_{50}$ to 3×10^{10} $TCID_{50}$ days 1-5 (dose escalation) with *IV* paclitaxel 175 mg/m^2 over 3 h and *IV* carboplatin AUC_5 (over 30 min) on day 1 every 21 d (3×10^{10} $TCID_{50}$ *IV* reovirus days 1-5 every 21 d became recommended phase Ⅱ dose with paclitaxel and carboplatin)	Out of 26 patients, best overall response was CR in 1 patient (3.8%,head and neck cancer), PR in 6 patients (23.1%, 3 each with SCC of head and neck and head and neck cancer), major clinical response not evaluable by RECIST criteria in 2 patients (7.7%, SCC of head and neck), and SD in 9 patients (34.6%, 3 with SCC of head and neck, 3 with head and neck cancer, 1 with gynecological cancer, 1 with melanoma, 1 with sarcoma) with median duration of SD and PR of 6 mo (range 3-10 mo). Of the 24 patients with head and neck cancer, median OS was 7.1 mo (CI: 4.2-11.5 mo)
Ⅰ/Ⅱ (OSU-07022, NCI trial)	Recurrent or refractory ovarian, peritoneal, and fallopian tube carcinomas	60-min *IV* infusion 3×10^{10} $TCID_{50}$ days 1-5 with daily IP administration days 2-3 beginning cycle 2 every 28 d (dose escalation with IP dosing)	Thus far 8 patients have received treatment. Biopsied ovarian and peritoneal tumor samples reveal detection of viral proteins in tumor tissues compared to control after systemic (*IV*) administration of reovirus and presence of reovirus replication in tumors due to overlap of reovirus protein and microtubules
Ⅱ (REO 008)	Various advanced or refractory solid malignancies	Open-label, single-arm, multicenter: 1×10^{10} $TCID_{50}$ intratumoral injection on days 2 and 4 with 4 Gy local irradiation daily × 5 (total 20 Gy) every cycle	Out of 16 patients enrolled (5 with melanoma, 4 colorectal, 1 gastric, 1 ovarian, 1 pancreatic, 1 lung, 1 cholangiocarcinoma, 1 sinus, 1 thyroid), 14 were evaluable and best overall response was SD or better in 13 patients (93%). Of these patients, 4 had PR (2 with melanoma, 1 lung, 1 gastric) and 2 had minor responses (1 thyroid and 1 ovarian)
Ⅱ (MAYO-MC0672, NCI trial)	Metastatic melanoma	Open-label, single-arm, multicenter: 60-min *IV* infusion 3×10^{10} $TCID_{50}$ days 1-5 every 28 d	Out of 21 evaluable patients, best overall response was SD > 8 wk in 6 patients. The median TTP was 45 d (range 13-96 d) and median OS was 165 d (range 15 d-15.8 mo). Trial was closed as did not meet previously defined efficacy criteria to proceed to second stage of accrual
Ⅱ (REO 014)	Advanced or refractory sarcomas metastatic to lung	Open-label, single-arm, multicenter: 60-min *IV* infusion 3×10^{10} $TCID_{50}$ days 1-5 every 28 d	Out of 53 enrolled patients, best overall response was SD ≥ 12 wk in 18 patients (34%) with a subgroup of 12 patients (3 with synovial sarcoma, 2 with leiomyosarcoma, 2 with MFH, 1 with ES, 1 with non-specified spindle cell sarcoma, 1 with chordoma, 1 with ASPS), 1 with myxoid liposarcoma) having prolonged SD > 16 wk. Three of these patients demonstrated SD > 1 yr (1 with MFH, 1 with synovial sarcoma, 1 with ES). The median TTP was 58.0 d (95%CI, 54-110, range 8-726 d). The prolonged SD demonstrated fulfilled the study criteria for consideration as an active agent
Ⅱ (REO 015)	Refractory, recurrent, or metastatic SCC of the head and neck	Open-label, single-arm: 60-min *IV* infusion 3×10^{10} $TCID_{50}$ days 1-5 with *IV* paclitaxel 175 mg/m^2 over 3 h and *IV* carboplatin AUC_5 (over 30 min) on day 1 every 21 d	Out of 13 evaluable patients (sites included 3 larynx, 6 oral cavity, 4 pharynx, 1 other), 4 had PR (31%) and 2 had SD ≥ 12 wk for a CBR of 46%
Ⅱ (REO 016)	Recurrent or metastatic NSCLC	60-min *IV* infusion 3×10^{10} $TCID_{50}$ days 1-5 with *IV* paclitaxel 175 mg/m^2 over 3 h and *IV* carboplatin AUC_5 (over 30 min) on day 1 every 21 d	Out of 37 patients enrolled, 20 patients had detected K-Ras mutations, 3 patients had EGFR mutations, 10 patients had EGFR amplifications alone, and 4 patients had BRAF V600E mutations. Median PFS was 4 mo (95%CI: 2.9-6.1), median OS was 13.1 mo (95%CI: 9.2-21.6), and 1-yr OS rate was 57% (95%CI: 39%-72%)
Ⅱ (REO 017)	Advanced or unresectable pancreatic cancer	60-min *IV* infusion 1×10^{10} $TCID_{50}$ on days 1, 2, 8 and 9 with *IV* infusion of gemcitabine 800 mg/m^2 days 1 and 8 every 21 d	Out of 34 enrolled patients, median PFS was 4 mo and OS was 10.2 mo. One- and 2-yr survival rates were 45% and 24%, respectively
Ⅱ (REO 021)	Recurrent or metastatic SCC of the lung	Open-label, single-arm: 60-min *IV* infusion 3×10^{10} $TCID_{50}$ days 1-5 with *IV* paclitaxel 200 mg/m^2 over 3 h and *IV* carboplatin AUC_6 every 21 d	Out of 25 patients who received more than 1 cycle of therapy, best overall response was PR in 12 patients (48%) and SD in 10 patients (40%) for a CBR of 88%. Of 21 patients with > 6 mo follow-up 7 had PFS ≥ 6 mo (33.3%)
Ⅲ (REO 018)	Advanced or metastatic head and neck cancer	Randomized, double-arm, double-blinded, multicenter: 60-min *IV* infusion 3×10^{10} $TCID_{50}$ days 1-5 with standard doses of *IV* paclitaxel and *IV* carboplatin on day 1 only every 21 d (treatment arm) *vs* standard doses of *IV* paclitaxel and *IV* carboplatin alone (control arm)	Out of 167 enrolled patients, 118 patients were segregated into an intent-to-treat basis group with loco-regional head and neck cancer (with or without metastases). In this group, median PFS was 94 d (13.4 wk, n = 62) in the test arm *vs* 50 d (7.1 wk, n = 56) in control arm maintained through 5 cycles. In the 88 patients discontinued from the study from this group, median OS was 150 d (21.4 wk, n = 50) in the test arm *vs* 115 d (16.4 wk, n = 38) in the control arm. Survival analysis in the other group (distal metastases-only) has not been conducted

IV: Intravenous; $TCID_{50}$: Tissue culture infectious dose-50; $AUC_{5/6}$: Area under curve-5/-6; CR: Complete response; PR: Partial response; SCC: Squamous cell carcinoma; RECIST: Response evaluation criteria in solid tumors; SD: Stable disease; OS: Overall survival; IP: Intraperitoneal; Gy: Gray; TTP: Time to disease progression; PFS: Progression-free survival; MFH: Malignant fibrous histiocytoma; ES: Ewing sarcoma; ASPS: Alveolar soft part sarcoma; CBR: Clinical benefit rate; NSCLC: Non-small cell lung cancer; EGFR: Epidermal growth factor receptor.

included pyrexia (43.5%), lymphopenia (26.1%), and influenza-like symptoms (17.4%)[73]. The best overall response was PR in 2 patients and SD in 5 patients (out of 7 patients in phase Ⅰa) and PR in 5 patients and

SD in 2 patients (out of 7 patients in phase Ⅰb) up to 3 mo post-treatment[73]. The recommended phase II doses were 1 × 10^{10} $TCID_{50}$ of reovirus × 2 intratumoral injections with 20 Gy of radiation × 5 fractions and 1 × 10^{10} $TCID_{50}$ of reovirus × 6 intratumoral injections with 36 Gy of radiation × 12 fractions for short and prolonged palliative regimens, respectively[73]. Another phase Ⅰ study (REO 009) originally used 3 × 10^9 $TCID_{50}$ days 1-5 of *IV* reovirus with gemcitabine in the treatment of advanced solid tumors but the dosing of reovirus was amended to 1 × 10^9 $TCID_{50}$ to 3 × 10^{10} $TCID_{50}$ *IV* reovirus on day 1 only with 30-min *IV* infusion of gemcitabine 1000 mg/m^2 on days 1 and 8 every 21 d when DLTs of grade 3 transaminitis and grade 3 elevation in troponin Ⅰ occurred[74]. A MTD was not reached, but a third DLT of grade 3 transaminitis also occurred at the amended 3 × 10^{10} $TCID_{50}$ day 1 dose[74]. Interestingly, the elevation in liver enzymes was associated with concomitant acetaminophen use and prompted the recommendation of avoidance of acetaminophen during reovirus clinical trials[74]. The most common treatment-related AEs were pyrexia (68.8%), nausea (43.8%), and diarrhea (37.5%), and 1 × 10^{10} $TCID_{50}$ *IV* reovirus on day 1 became the recommended phase Ⅱ dose in combination with gemcitabine[74]. Out of 10 patients, the best overall response was PR after 4 cycles in 2 patients and SD from 4-8 cycles in 5 patients (median SD of 72 d, range 36-112 d) for a CBR of 80%[74].

In REO 010, a MTD was not reached though a DLT of grade 4 neutropenia resulted in a 20% reduction of the docetaxel dose in refractory or metastatic solid cancers treated with 3 × 10^9 $TCID_{50}$ to 3 × 10^{10} $TCID_{50}$ days 1-5 of *IV* reovirus (the last being the recommended phase Ⅱ dose with docetaxel) with *IV* docetaxel 75 mg/m^2 on day 1 every 21 d[75]. Four AEs of grade 4 neutropenia were felt to be due to docetaxel alone and an additional grade 4 lymphopenia also occurred; the most common AEs were flu-like symptoms, diarrhea, and fatigue[75]. Out of 16 patients, the best overall response was PR ⩾ 2 cycles in 4 patients and SD ⩾ 2 cycles in 10 patients for a CBR of 88%[75]. REO 013 enrolled 10 patients with metastatic colorectal cancer to the liver to be treated with 1 × 10^{10} $TCID_{50}$ of *IV* reovirus daily × 5 d between 6-28 d prior to planned radical resection of liver metastases[76]. There were no grade 3 or higher toxicities and the most common AEs were flu-like symptoms[76]. Resected tumor specimens from 9 patients showed staining for reovirus protein greatest in tumor when compared to surrounding tumor stroma and normal liver[76].

Preliminary results of REO 022 included PR in 1 patient (5%) and SD in 9 patients (50%) with a median progression-free survival (PFS) in irinotecan/fluorouracil/leucovorin (FOLFIRI)-naïve patients of 7.4 mo (95%CI: 1.9-12.9 mo) and overall median PFS of 7.4 mo (95%CI: 0.6-14.1 mo) in 18 patients with metastatic colorectal cancer treated with 60-min *IV* infusion of reovirus from 1 × 10^{10} $TCID_{50}$ to 3 × 10^{10} $TCID_{50}$ days 1-5 every 28 d with standard FOLFIRI[77]. Irinotecan 150 mg/m^2 with 3 × 10^{10} $TCID_{50}$ *IV* reovirus days 1-5 every 28 d became the recommended phase Ⅱ dose[77]. The most common (> 10%) grade 3 or higher toxicities were neutropenia, anemia, and thrombocytopenia, and DLTs of neutropenia were observed[77].

Results from a National Cancer Institute (NCI)-sponsored phase Ⅰ study (OSU-11148) included SD in 5 of 12 patients (42%) with relapsed multiple myeloma treated with 60-min *IV* infusion of reovirus from 3 × 10^9 $TCID_{50}$ to 3 × 10^{10} $TCID_{50}$ days 1-5 every 28 d[78]. A MTD was not reached, no DLTs were observed, and grade 3 toxicities included neutropenia, leukopenia, thrombocytopenia, and hypophosphatemia (Table 1)[78]. From this study, combination therapy is presumed to be more beneficial than oncolytic reovirus therapy alone in patients with multiple myeloma. Overall, phase Ⅰ trials did demonstrate that treatment with reovirus *via* various methods of administration was well tolerated by patients with minimal adverse effects.

Phase Ⅰ/Ⅱ trials

A phase Ⅰ/Ⅱ trial (REO 011) involved 60-min *IV* infusion of reovirus from 3 × 10^9 $TCID_{50}$ to 3 × 10^{10} $TCID_{50}$ days 1-5 (the latter being the recommended phase Ⅱ dose) with *IV* paclitaxel 175 mg/m^2 over 3 h and *IV* carboplatin area under curve-5 (AUC_5) over 30 min on day 1 every 21 d in untreatable, relapsed, or metastatic solid cancers[79]. A MTD was not reached even at ceiling doses and there were no DLTs observed though a total of 8 patients required dose reductions in paclitaxel and carboplatin[79]. The most common treatment-related AEs were alopecia (64.5%), fever (58.1%), and fatigue (58.1%); no relationships between reovirus dose and incidence or grade of symptoms were observed[79]. Out of 26 patients, the best overall response was CR in 1 patient (3.8%), PR in 6 patients (23.1%), major clinical response not evaluable by standard criteria in 2 patients (7.7%), and SD in 9 patients (34.6%) with a median duration of SD and PR of 6 mo (range 3-10 mo)[79]. Of the 24 patients with head and neck cancer, the median OS was 7.1 mo (CI: 4.2-11.5 mo)[79]. Preliminary results from a NCI-sponsored trial (OSU-07022) showed penetration and detection of replicating reovirus in tumor tissues thus far in 8 patients with recurrent or refractory ovarian, peritoneal, and fallopian tube carcinomas treated with *IV* reovirus at a fixed dose of 3 × 10^{10} $TCID_{50}$ days 1-5 with dose-escalation of daily intraperitoneal (IP) reovirus every 28 d (Table 2)[80,81].

Pharmacokinetics and pharmacodynamics

In keeping with the wide range of historically observed seropositivities to reovirus, baseline seropositivity for NARAs was 37% in one phase Ⅰ study and more than 90% in another phase I trial[68,82]. In general, phase Ⅰ trials demonstrated a wide time to induction and time to peak levels of NARA titers from baseline though both more or less occurred within 1-4 wk with a median time to induction of 1.4 wk (range 1-3 wk) and median time to peak of 3.8 wk (range 1-10 wk) in one study[42,68,69,71-76,78,79]. Maximum NARA titers also

varied considerably from 1/512 in one study to greater than 1/531441 in another (expressed as last dilution causing < 80% cytotoxicity) with a median increase from baseline of 250-fold (range 9- to 6437-fold)[42,72,82]. The neutralizing antibody response appeared to be blunted in cohorts with leukopenia from high-dose systemic reovirus therapy and myelosuppression from prior lumbosacral or pelvic radiotherapy[82]. Interestingly, reovirus in combination with gemcitabine or paclitaxel/carboplatin resulted in an attenuation in the time to induction and peak levels of NARA titers compared to prior phase Ⅰ results while co-administration with docetaxel had no such effects[74,75,79]. Pharmacokinetic parameters, however, of gemcitabine, docetaxel, or paclitaxel/carboplatin, when co-administered with reovirus, were not appreciably different compared to receiving those agents alone[74,75,79]. Phase Ⅰ data also illustrated that reverse transcription-polymerase chain reaction (RT-PCR) analysis of specimens including serum, stool, urine, saliva, and sputum for post-treatment viral shedding were negative in a majority of cases highlighting that reovirus administration in the outpatient setting is relatively safe[42,68,69,71-73,75,79]. When post-treatment viral shedding RT-PCR analyses were positive, they generally occurred within a few weeks (range 1-149 d) with some exceptions[42,68,69,71-73,75,79]. REO 013 showed that viral genome, though replication-incompetent, was present in plasma in 80% of patients at 1 h after the first dose of reovirus[76]. However, replication-competent reovirus was detected in peripheral blood mononuclear cells (PBMCs), granulocytes, and platelets, but not in plasma and red blood cells, at 1-h post-infusion and as late as 5-d post-infusion in PBMCs highlighting the idea of reovirus "hitchhiking" on such cells to evade the NARA response[76].

With respect to pharmacodynamics, available pathologic specimens have demonstrated positive detection of reovirus proteins localized to areas of cancer (occasionally with less involvement of surrounding tumor stroma and adjacent areas of normal tissue) with evidence of reovirus replication, apoptosis, and necrosis consistent with cytopathic effects[42,71-73,75,76,78,80,81]. In REO 005, 3 patients had reductions in cancer markers (carcinoembryonic antigen and PSA) consistent with clinical benefit, and 3 patients with biopsies showed the presence of viable reovirus post-treatment whose recovered titers correlated with doses of reovirus administered[72]. Similarly, in REO 013, replicating virus was recovered from lysates from surgical specimens in all 4 patients tested[76]. Interestingly, patients with 100% co-expression of reovirus RNA and CD138 showed greatest reductions in percent of myeloma cells with treatment in a NCI-sponsored phase Ⅰ study (OSU-11148)[78].

Pathologic specimens in REO 002 showed peritumoral inflammation in 4 patients while REO 003 demonstrated focal collections of plasma cells not present previously during pathologic tumor examination in 3 of 6 patients[42,69]. Indeed, these observations have been somewhat corroborated in a separate phase Ⅰ trial (REO 005) that revealed increases in $CD3^+CD4^+$ T-lymphocytes in 47.6% of patients, $CD3^+CD8^+$ T-lymphocytes in 33% of patients, $CD8^+$ perforin/granzyme$^+$ T-lymphocytes in 23.8% of patients, $CD3^-CD56^+$ natural killer (NK) cells in 28.6% of patients, and combined T-cell helper 1 and 2 (Th1 and Th2) cytokines in 38% of patients after reovirus therapy highlighting the potential significance of immune-mediated responses as a facilitator of reovirus anticancer efficacy[82]. Of note, there were no clear relationships between immune responses and reovirus dose, clinical response, or toxicity[82].

Phase Ⅱ and Ⅲ trials

An early multicenter, single-arm, open-label, phase Ⅱ trial (REO 008) involved 1×10^{10} $TCID_{50}$ intratumoral injections of reovirus on days 2 and 4 with 4 Gy of local irradiation daily × 5 fractions (total 20 Gy per cycle) in the treatment of refractory or metastatic solid tumors[83]. Out of 14 evaluable patients, the best overall response was SD or better in 13 patients (93%)[83]. Of these 13 patients, 4 experienced PR (2 with melanoma, 1 with lung cancer, and 1 with gastric cancer) and 2 experienced minor response (1 with thyroid cancer and 1 with ovarian cancer)[83]. The most common treatment-related AEs were grade 1 or 2 chills, pyrexia, headache, lethargy, anorexia, vomiting, shivering, nausea, and mild pain at injection site[83]. The NCI-sponsored MAYO-MC0672 was a multicenter, single-arm, open-label, phase Ⅱ trial pitting 60-min *IV* infusion of reovirus 3×10^{10} $TCID_{50}$ days 1-5 every 28 d against metastatic melanomas[84]. Out of 21 evaluable patients, the best overall response was SD > 8 wk in 6 patients with a median TTP of 45 d (range 13-96 d) and median OS of 165 d (range 15 d-15.8 mo)[84]. The study was ultimately closed due to failure to meet previously defined efficacy criteria to proceed to second stage of accrual, but 1 patient with 2 surgically removed metastatic cutaneous lesions demonstrated treatment effect as 75%-90% necrosis of these lesions were present on sampling[84]. Of note, out of 13 biopsies with metastatic tumor, productive reovirus replication was detected in 2 patients who had longer PFS of 80 and 87 d, respectively[84]. No dose reductions occurred, and the most common treatment-related grade 1 or 2 AEs were fatigue (66.7%), nausea (57.1%), and fever (52.4%)[84]. The most common treatment-related grade 3 or 4 AEs were fatigue (9.5%), hyponatremia (9.5%), and lymphopenia (9.5%)[84].

REO 014 enrolled 53 patients with refractory or untreatable soft tissue and bone sarcomas metastatic to the lung in a multicenter, single-arm, open-label phase Ⅱ trial with 60-min *IV* infusions of reovirus 3×10^{10} $TCID_{50}$ administered on days 1-5 every and given 28 d (personal communication). The best overall response was SD ≥ 12 wk in 18 patients (34%) with a subgroup of 12 patients having prolonged SD > 16 wk. Of these 12 patients, 3 patients demonstrated SD > 1 year (1 with malignant fibrous histiocytoma, 1 with synovial sarcoma, and 1 with ES). The median TTP was 58.0 d

(95%CI: 54-110, range 8-726 d). The prolonged SD demonstrated fulfilled study criteria for consideration as an active agent. No dose reductions occurred, and the most common treatment-related AEs were pyrexia (81.1%), chills (66.4%), fatigue (47.2%), myalgia (37.7%), and nausea (37.7%). Of note, the first case of optic neuritis related to reovirus therapy was reported as a serious AE. Results from a single-arm, open-label, phase Ⅱ study (REO 015) were PR in 4 patients (31%) and SD ≥ 12 wk in 2 patients for a CBR of 46% in 13 patients with refractory, recurrent, or metastatic SCC of the head and neck treated with 60-min *IV* infusion of reovirus 3×10^{10} $TCID_{50}$ days 1-5 with IV paclitaxel 175 mg/m^2 over 3 h and IV carboplatin AUC_5 over 30 min on day 1 every 21 d[85]. Grade 1 or 2 AEs included fevers, chills, fatigue while grade 3 or 4 AEs were hypokalemia, fatigue, nausea, aspartate aminotransferase elevation, neutropenia, and anemia[85].

REO 016 enrolled 37 patients with recurrent or metastatic NSCLC originally treated with *IV* reovirus in combination with *IV* paclitaxel 200 mg/m^2 and *IV* carboplatin AUC_6, but due to grade 3 diarrhea and febrile neutropenia (1 each), the dosing regimen was amended to 60-min *IV* infusion of reovirus 3×10^{10} $TCID_{50}$ days 1-5 with *IV* paclitaxel 175 mg/m^2 over 3 h and *IV* carboplatin AUC_5 (over 30 min) on day 1 every 21 d[86]. Of note, 20 patients had detected K-*Ras* mutations, 3 patients had EGFR mutations, 10 patients had EGFR amplifications alone, and 4 patients had BRAF V600E mutations[86]. Updated results have shown a median PFS of 4 mo (95%CI: 2.9-6.1), median OS of 13.1 mo (95%CI: 9.2-21.6), and 1-year OS rate of 57% (95%CI: 39%-72%)[86]. The most common AEs were fatigue, diarrhea, nausea, arthralgia/myalgia, and anorexia[86]. Results from REO 017 have thus far included a median PFS of 4 mo and OS of 10.2 mo in 34 enrolled patients with advanced or unresectable pancreatic cancer treated with 60-min *IV* infusion of reovirus 1×10^{10} $TCID_{50}$ on days 1, 2, 8 and 9 with IV gemcitabine 800 mg/m^2 days 1 and 8 every 21 d[87]. Treatment was well tolerated with manageable non-hematologic toxicities including grade 3-4 asthenia (38%), fever (12%), diarrhea (9%), chills (3%), flu-like syndrome (3%), and nausea/vomiting (3%). Intriguingly, upregulation of immune checkpoint markers including programmed death-ligand 1 (PD-L1) on immunohistochemistry (IHC) was demonstrated following treatment with reovirus[87].

The open-label, single-arm phase Ⅱ trial (REO 021) involved 60-min *IV* infusion of reovirus 3×10^{10} $TCID_{50}$ days 1-5 with *IV* paclitaxel 200 mg/m^2 over 3 h and *IV* carboplatin AUC_6 every 21 d in the treatment of recurrent or metastatic SCC of the lung[88]. Out of 25 patients who received more than 1 cycle of therapy, the best overall response was PR in 12 patients (48%) and SD in 10 patients (40%) for a CBR of 88%[88]. Of the 21 patients with > 6 mo follow-up, seven patients experienced PFS ≥ 6 mo (33.3%)[88]. The most common AEs were those expected of paclitaxel/carboplatin including neutropenia and thrombocytopenia and those expected of reovirus such as fever and fatigue[88]. The only treatment-related serious AE was reversible grade 2 elevation in creatinine and blood urea nitrogen[88].

On November 21, 2013, Oncolytics Biotech® Inc. reported preliminary top-line data from the randomized, double-arm, double-blinded, multicenter phase Ⅲ trial involving 60-min *IV* infusion of reovirus 3×10^{10} $TCID_{50}$ days 1-5 with standard doses of *IV* paclitaxel and IV carboplatin on day 1 only every 21 d (test arm) *vs* standard doses of *IV* paclitaxel and *IV* carboplatin alone (control arm) in the treatment of advanced or metastatic head and neck cancers (http://www.oncolyticsbiotech.com/clinical-trials). Per their report, 167 patients were enrolled and divided into an intent-to-treat group of 118 patients with loco-regional head and neck cancer (with or without metastases) and another group with distal metastases only. In the group of 118 patients, the median PFS was 94 d (13.4 wk, *n* = 62) in the test arm *vs* 50 d (7.1 wk, *n* = 56) in control arm maintained through 5 cycles. In the 88 patients discontinued from the study from this group, median OS was 150 d (21.4 wk, *n* = 50) in the test arm *vs* 115 d (16.4 wk, *n* = 38) in the control arm. Survival analysis in the distal metastases-only group has not been conducted. Of note, at the time of the first post-treatment scan (post-cycle 2 of therapy), 62 patients in the test arm experienced PD (32.3%) *vs* 56 patients on the control arm (51.8%, *P* = 0.04), and of the 86 patients with measurable disease at the first post-treatment scan, 48 patients demonstrated tumor reduction in the test arm *vs* 38 patients in the control arm (*P* = 0.049). There was a statistically significant increase in AEs of fever, chills, nausea, and diarrhea in the test arm *vs* control arm though there were no statistical differences in hematologic parameters in both arms. Nine patients in each arm experienced serious AEs of neutropenia with or without demonstrated infection. Interestingly, there were no dose reductions of paclitaxel for neuropathy or neurotoxicity in the test arm *vs* 6 dose reductions in the control arm (*P* = 0.028, Table 2).

IMMUNE RESPONSES TO REOVIRUS

Neutralization by the host immune system

Early preclinical evidence showed that prior exposure to reovirus did not significantly limit the antitumor activity of locally administered (intratumoral) reovirus in immune-competent C3H mice implanted with *Ras*-transformed fibroblasts and previously challenged with intramuscular injection of reovirus (detection of reovirus antibodies occurred after 2 wk in all challenged animals)[20]. Neutralizing antibodies similarly did not affect the efficacy of intratumoral reovirus in immune-competent rodent models of subcutaneous and intracranial glioblastoma[89]. However, systemic administration (*IV*) of reovirus is of therapeutic importance in advanced cancers, and phase Ⅰ data illustrated that even heavily pretreated patients were capable of mounting brisk and dynamic immune responses to *IV* reovirus characterized by peak

NARA titers reached by day 7 in 37.5% of patients and by day 14 in 62.5% of patients[82]. Indeed, neutralization of systemic reovirus by the host immune system was demonstrated when immune-competent C3H mice bearing *Ras*-transformed fibroblastic tumor allografts treated with *IV* reovirus (*via* tail vein injections) exhibited significant inhibition of tumor growth compared to controls at first, but tumor regrowth occured by 3 wk of *IV* reovirus therapy which coincided with rising serum NARA titers[58]. The ability of systemic reovirus to suppress tumor growth in immunized mice, however, was restored when co-administered with immunosuppressive agents such as cyclosporin A or cyclophosphamide which correlated with significantly decreased production of NARAs comparable to levels in mice without previous exposure to reovirus[58].

Systemic reovirus carries an innate ability to evade the NARA response by "hitchhiking" in PBMCs, granulocytes, and platelets; this process is detectable within a few hours post-infusion[82]. However, to further counteract the significant barrier to efficacy imposed by the neutralizing antibody response, it has been recommended that systemic reovirus be administered in rapid, repeated, and high doses within the first week of treatment when the NARA response has yet to become amplified[82]. Another strategy has involved the combination of reovirus with chemotherapeutic, particularly immunosuppressive, agents that attenuate the NARA response and therefore enhance tumor seeding of the virus, as previously suggested and described[66]. Importantly, early phase clinical trials have demonstrated that reovirus in combination with gemcitabine or paclitaxel/carboplatin resulted in attenuation of the NARA response while co-administration with docetaxel had no such effects though this finding was inconsistent with preclinical data[66,74,75,79]. All 3 combination regimens, however, have produced promising findings of clinical efficacy in various advanced malignancies and await further investigation in later trials[74,75,79].

Protective function against reovirus toxicities

High-dose systemic reovirus therapy is not without inherent risks as mice killed by viral overdose showed pathologic changes among several organs including liver and heart[58]. The role of the immune system in protecting against reovirus toxicity was highlighted when reovirus co-administered with high-dose cyclophosphamide resulted in both undetectable levels of NARA titers and severe systemic toxicities characterized by myocarditis, liver necrosis, tail detachment, and death compared to controls[56]. Furthermore, reovirus has been associated with limb necrosis and death in approximately 50%-60% of reovirus-treated SCID mice[20]. Upon metronomic dosing of high-dose cyclophosphamide with reovirus, however, systemic toxicities were markedly reduced in the presence of detectable NARA titers while preserving high levels of tumor access to virus and antitumor efficacy[56].

In a phase Ⅰ, dose escalation trial cyclophosphamide was co-administered with reovirus in 36 patients with various solid tumors that had received prior therapies. The dose of cyclophosphamide ranged from 25-1000 mg/m^2 with at least 3 patients per cohort with a consistent dose of reovirus dose of 3×10^{10} $TCID_{50}$/d[90]. The combination of cyclophosphamide and reovirus was well tolerated with few grade 3 toxicities including fever, diarrhea, neutropenia, and anemia[90]. However, cyclophosphamide did not have an effect at stimulating an antiviral response as NARA titers rose > 50 fold in all but one patient[90]. Interestingly, reoviral RNA was detected *via* RT-PCR in PBMCs despite the significant rise in NARA titers, suggesting that PBMCs play a role in viral delivery to tumor cells[90].

Immune-mediated antitumor activity of reovirus

It has long been postulated that oncolytic virotherapy stimulates antitumor immune responses through innate and adaptive pathways[91]. Accordingly, several investigations have shown that reovirus infection: (1) induces the release of a host of pro-inflammatory mediators including interleukin (IL)-1α, IL-1β, IL-2, IL-6, IL-8, IL-12p40/70, IL-17, regulated on activation, normal T cell expressed and secreted, macrophage inflammatory protein-1α/β, granulocyte macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-α, IFN-γ, and tumor necrosis factor-α; (2) suppresses the release of the immunosuppressive IL-10; and (3) increases activation of dendritic cells (DCs) and recruits effectors from both innate and adaptive immunity including cytotoxic CD8$^+$ T-lymphocytes (CTLs) and NK cells to facilitate tumor cell killing[35,92,93]. Furthermore, reovirus-infected melanoma cells released eotaxin, interferon gamma-induced protein 10, and IFN-β, in a NF-κB and PKR-dependent manner, and recruited NK cells, DCs, and CTLs to altogether promote bystander immune-mediated cytotoxicity in the tumor microenvironment[94].

Although reovirus infection has been shown to induce DC maturation in a dose-dependent manner, the immune-mediated antitumor activity of reovirus appears to occur independent of direct viral oncolysis or replication[95,96]. Nevertheless, reovirus therapy is capable of stimulating pro-inflammatory responses, enhancing tumor antigen presentation and exposing inaccessible tumor antigens for processing by DCs and CTLs, overcoming tumor evasion strategies and priming adaptive tumor-specific T-cells *in vitro* and *in vivo*, and initiating antitumor immunity to protect against subsequent tumor challenges in an antigen-dependent but reovirus-independent manner[92,96]. These processes that orchestrate reovirus-mediated antitumor immune responses have been demonstrated, in part, across several cancers including melanoma, lung cancer, AML, and prostate cancer[46,92,97]. Importantly, further support has been offered in early clinical trials when phase Ⅰ data showed increases in CD3$^+$CD4$^+$ T-lymphocytes in 47.6% of patients, CD3$^+$CD8$^+$ T-lymphocytes in 33% of patients, CD8$^+$perforin/granzyme$^+$ T-lymphocytes in 23.8% of patients, CD3-CD56$^+$ NK cells in 28.6% of

patients, and combined Th1 and Th2 cytokines in 38% of patients after reovirus therapy[82].

Interestingly, administration of reovirus with tumor-specific DCs or OT-1 T-cells in melanoma-bearing mice resulted in significantly higher survival rates compared to controls and highlighted the synergistic potential of reovirus with immunotherapy[92]. Intratumoral reovirus co-administered with intraperitoneal genetically modified cells expressing IL-2, IL-12, or GM-CSF in mice inoculated with TC-1 cancer cells failed to demonstrate significant synergistic effects with respect to tumor suppression though the combination of reovirus with cyclophosphamide (administered at specific time points) produced synergistic inhibition of tumor growth[98]. Preconditioning of mice bearing subcutaneous melanomas with regulatory T-cell (Treg) depletion and IL-2 significantly enhanced the delivery of *IV* reovirus to tumors and increased antitumor efficacy compared to controls though with severe systemic toxicities such as shortness of breath, inactivity, and tail necrosis/detachment[99]. Instead, preconditioning with cyclophosphamide and IL-2, which mimicked Treg depletion, induced "hyperactivated" NK cells and similarly enhanced antitumor efficacy with *IV* reovirus though without detectable toxicities[99]. Alternatively, reovirus in combination with gemcitabine in mice implanted with ovarian cancer cells demonstrated greater survival and postponement of peritoneal carcinomatosis by inhibiting myeloid-derived suppressor cells (MDSCs), downregulating pro-MDSC factors, and accelerating tumor-specific T-cell responses[100].

Also of relevance, recent phase Ⅱ trials have identified prolonged OS with reovirus in combination with conventional chemotherapy in advanced NSCLC and pancreatic cancer suggestive of an immunomodulatory influence on outcomes[86,87]. Upregulation of the immune checkpoint marker PD-L1 on IHC was observed following treatment with reovirus in REO 017. Although immune checkpoint inhibition and boosting of the immune response may be counterintuitive and detrimental to the efficacy of oncolytic reovirus by restricting viral replication, reovirus therapy in combination with anti-PD-1 therapy demonstrated improved survival in mouse models of melanoma, *in vivo*, compared to reovirus or anti-PD-1 therapy alone[101]. Checkpoint inhibition improved the ability of NK cells to kill reovirus-infected tumor cells and enhanced the $CD8^+$ Th1 antitumor response primed by reovirus therapy *in vitro*. Furthermore, PD-1 blockade enhanced antiviral immune responses but through mechanisms that may differ from those affecting the antitumor response and thus offering a novel platform for combining immune modulation and reovirus in anticancer therapy.

DISCUSSION, PERSPECTIVES AND THE FUTURE

At the time of this review, there are a total of 34 clinical trials (both ongoing and completed) involving wild-type, unmodified T3D reovirus (Reolysin®) in the treatment of a variety of cancers (http://www.oncolyticsbiotech.com/clinical-trials). Nineteen of these clinical trials are early phase trials (phase Ⅰ and Ⅰ/Ⅱ) or translational studies, and 10 of these 19 trials (53%) have investigated reovirus as monotherapy. Although not the primary objectives of these early trials, several phase Ⅰ trials investigating single-agent reovirus produced promising results with a CBR as high as 45% in one study (though with a smaller and limited cohort of patients) when antitumor responses were evaluated by conventional criteria and reported (Table 1)[68,69,71,72,78]. However, of the remaining 15 clinical trials (phase Ⅱ and Ⅲ), only 2 of these investigated reovirus as monotherapy (13%). In an attempt to carry over the clinical efficacy observed in earlier trials, one phase Ⅱ trial investigating single-agent reovirus in metastatic melanomas (the NCI-sponsored MAYO-MC0672) failed to meet previously defined efficacy criteria to advance to second stage of accrual and was ultimately closed[84]. However, REO 014 is the only phase Ⅱ trial in which single-agent reovirus fulfilled study criteria for consideration as an active agent in untreatable, refractory, or metastatic sarcomas; further trials involving reovirus as montherapy in advanced sarcomas are warranted (personal communication). Nevertheless, this trend is likely a reflection of a growing consensus that single-agent reovirus is unlikely to have sufficient clinical efficacy to be used alone as an anticancer agent[2,4,6,7].

The delivery of viruses to target tissues in sufficient numbers to produce a meaningful therapeutic effect has been a longstanding tenet of virotherapy[2]. Early investigations into the anticancer potential of reovirus demonstrated that the neutralizing antibody response to the virus may pose a dilemma to its therapeutic efficacy given its ubiquitous nature and high seropositivity within the population. The effect of the neutralizing antibody response was most profound with systemic (*IV*) reovirus, which is of therapeutic importance in advanced cancers, when repeated *IV* delivery of reovirus in immune-competent mice bearing *Ras*-transformed tumor allografts demonstrated tumor regrowth within a few weeks that coincided with rising NARA titers[58]. Furthermore, phase Ⅰ data showed that even heavily pretreated patients experienced a brisk induction of NARA titers from baseline with a time to peak levels within a few weeks after systemic reovirus.

In an attempt to circumvent this barrier to efficacy, systemic reovirus has been administered in rapid, repeated, high doses within the first week of treatment before the NARA response is boosted. Another strategy involves improving tumor cell killing by including reovirus in combination with other anticancer therapies; this appears to be the avenue in which the majority of ongoing and future trials involving reovirus are headed. Reovirus offers an excellent toxicity profile with the most common treatment-related AEs being mild respiratory/enteric and constitutional symptoms characteristic of its viral pathophysiology. As a result, reovirus becomes

an attractive agent to use in combination with other therapies and, overall, makes combination clinical trials much more feasible. Furthermore, the mechanism of reovirus oncolysis offers synergistic potential when used with other agents due to differing pathways of inducing cancer cell death. These reasons have formed, in part, the rationale for late phase clinical trials, and so far, very promising preliminary results have been produced in several phase Ⅱ trials involving reovirus in combination with chemotherapy and radiotherapy with CBRs achieved as high as greater than 90% in one study (Table 2). Recently, updated results from REO 016 and 017 have demonstrated discordance between PFS and OS in advanced NSCLC and pancreatic cancer treated with reovirus in combination with conventional chemotherapy. The reported PFS in these trials are comparable to historical controls, but OS is substantially longer than what has ever been reported in the literature for both cancers[86,87]. The clear OS benefit in the face of apparently limited impact on PFS is often characteristic of immune involvement in outcomes and may suggest further immunomodulatory anticancer effects from reovirus therapy (see below). Preliminary results from the phase Ⅲ trial involving reovirus with paclitaxel and carboplatin in advanced head and neck cancer are also promising with improved median PFS and median OS when compared to control arms; the results of this trial are highly anticipated.

Independent of direct viral oncolysis and replication, reovirus offers further anticancer potential by promoting antitumor immune-mediated responses characterized by stimulation of pro-inflammatory cascades, activation of DCs, and recruitment of NK cells and CTLs that altogether contribute to bystander cytotoxicity within the tumor microenvironment. In addition, reovirus infection primes adaptive tumor-specific T-cell responses that can provide further tumor immunity and protection against subsequent challenges with tumor. Aside from the added cytotoxic effects offered with chemotherapy, certain agents may also enhance tumor seeding of reovirus due to attenuation of the NARA response as shown by gemcitabine, paclitaxel, and carboplatin. Immunomodulation with immunosuppressive agents such as cyclosporin A and cyclophosphamide also enhanced reovirus antitumor efficacy by attenuating NARAs but consequently revealed the protective function of the NARA response against reovirus systemic toxicity. The immune responses to reovirus, therefore, represent a double-edged sword in that they can pose a significant barrier to tumor seeding of virus and antitumor efficacy but also serve to protect against severe reovirus toxicity and promote antitumor cytotoxicity through innate and adaptive responses.

Despite the promising development of reovirus as an anticancer agent, there remain several key areas warranting further investigation in order to maximize the anticancer potential of reovirus. Firstly, a few reports have argued that reovirus oncolysis can occur independently of activated Ras and EGFR signaling pathways[43,44]. Despite the coordination between *Ras*-transformation, PKR, and viral translational inhibition, which remains one of the best characterized hypotheses in explaining the mechanism of reovirus oncolysis, greater understanding of the infectious life cycle of reovirus has uncovered that multiple steps of the oncolytic cycle including viral uncoating, production of viral progeny, progeny release through increased apoptosis, and spread of virus in later rounds of infection are influenced by *Ras*-transformation. Other studies have demonstrated potential ties between reovirus oncolysis and cell cycle phase[102]. These new insights have presented potential opportunities to enhance reovirus antitumor efficacy such as adding exogenous proteases to enhance reovirus infectivity, using Nutlin-3a to enhance reovirus-induced apoptosis and virus spread through p53-dependent NF-κB activation, using hydroxyurea to affect cell cycle synchronization and enhance sensitivity to reovirus, and avoiding agents that inhibit microtubules as functional microtubules are required for reovirus endocytic processing and infectivity[102-105]. Recent studies demonstrated that cancer-upregulated gene 2 inhibits PKR activation but is still dependent on p38 and Ras activation for permissiveness to reovirus replication, which highlights the increasing complexity and degree of crosstalk evident between mediators in coordinating sensitivity to reovirus oncolysis[106]. Undoubtedly, the mechanism of reovirus oncolysis in relation to EGFR/Ras activated signaling pathways (both upstream and downstream), PKR, the reovirus life cycle, cell cycle phase, and pathways of cell death warrant further investigation.

Future studies will also need to elucidate methods to promote antitumor immune responses while suppressing immune responses against tumor seeding of reovirus without severe systemic toxicities. Immunomodulation with preconditioning with cyclophosphamide and IL-2 has shown to enhance systemic delivery of reovirus and antitumor efficacy with reduced toxicities[99]. Future trials involving reovirus in combination with immunotherapy are warranted and likely to grow in number. Phase Ⅰ data involving reovirus and cyclophosphamide in advanced malignancies will likely provide greater insight in how to safely maximize reovirus-mediated antitumor immune responses while minimizing the immune responses against tumor targeting. Checkpoint inhibition represents an alternative, but increasingly popular, means for combining immunomodulation with reovirus as anti-cancer therapy. Preclinical studies have demonstrated improved anticancer efficacy with the combination of PD-1 blockade and reovirus therapy compared to either therapy alone. Although antiviral responses were enhanced with the addition of anti-PD-1 therapy, they appear to occur through pathways that may differ from those affecting the antitumor response. Furthermore, checkpoint inhibition improved T-cell antitumor responses primed by reovirus therapy and the ability to locally clear reovirus-infected tumor cells. Indeed, with the growing popularity of checkpoint inhibitors in the treatment of

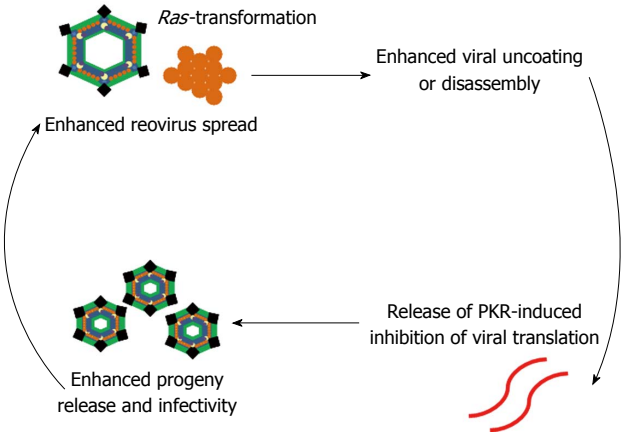

Figure 1 ***Ras*-transformation promotes reovirus preferential replication in cancer cells or oncolysis by affecting several key steps of the viral infectious life cycle.** *Ras*-transformation enhances viral uncoating or dissembly. dsRNA-activated protein kinase (PKR), which in the presence of viral transcripts, normally phosphorylates eukaryotic initiation factor 2α rendering it inactive and thereby leading to the inhibition of protein synthesis and viral replication, remains inactivated in *Ras*-transformed cells. Lastly, *Ras*-transformation enhances generation of viral progeny with increased infectivity, enhances release of progeny through apoptosis, and enhances viral spread in subsequent rounds of infection.

advanced cancers, clinical trials with immunomodulation and reovirus should be a focus of future studies. Upregulation of the immune checkpoint marker PD-L1 on IHC has also been observed following treatment with reovirus in REO 017. Whether increased levels of PD-L1 affect response to checkpoint inhibitors and reovirus therapy represents another issue in need of further investigation.

Recent developments highlight that reverse genetics and classical genetics have allowed for the engineering of genetically modified variants of reovirus that maintain or even enhance selective oncolytic potency while reducing toxicity[107-110]. Lastly, immune resistance to one particular oncolytic virus may not necessarily confer resistance to others, and combination therapies including multiple oncolytic viruses are possible as exemplified by the preclinical success of reovirus in combination with Newcastle disease virus or parvovirus in glioblastomas[65].

CONCLUSION

Reovirus is a dsRNA virus with demonstrated preferential replication in cancer cells, or oncolysis. The mechanism of reovirus oncolysis is still poorly understood though *Ras*-transformation and activated Ras signaling, appears central for sensitivity to reovirus replication. *Ras*-transformation modulates several steps of the viral life cycle in promoting reovirus oncolysis: (1) virus disassembly and uncoating; (2) releasing translational inhibition by PKR; (3) generation of infectious progeny; (4) enhanced apoptosis and progeny release; and (5) spread of virus in subsequent cycles of infection (Figure 1). The antitumor efficacy of reovirus is also largely dependent on immune-mediated antitumor effects involving both innate and adaptive responses. Wild-type, unmodified, replication-competent T3D reovirus (Reolysin®) has demonstrated anticancer activity across a spectrum of malignancies. Early clinical trials have shown a safe and tolerable toxicity profile of reovirus with a predictable NARA response, minimal viral shedding, and localization, replication, and cytotoxic effects in pathologic specimens consistent with activity. Phase Ⅱ and Ⅲ trials involving reovirus have demonstrated promising results of clinical efficacy and reinforce its potential as an anticancer agent. Future trials will likely take advantage of its excellent toxicity profile in combination therapies for synergistic tumor cell killing.

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REVIEW

Oncogenic role of leptin and Notch interleukin-1 leptin crosstalk outcome in cancer

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Abstract

Obesity is a global pandemic characterized by high levels of body fat (adiposity) and derived-cytokines (*i.e.*, leptin). Research shows that adiposity and leptin provide insight on the link between obesity and cancer progression. Leptin's main function is to regulate energy balance. However, obese individuals routinely develop leptin resistance, which is the consequence of the breakdown in the signaling mechanism controlling satiety resulting in the accumulation of leptin. Therefore, leptin levels are often chronically elevated in human obesity. Elevated leptin levels are related to higher incidence, increased progression and poor prognosis of several human cancers. In addition to adipose tissue, cancer cells can also secrete leptin and overexpress leptin receptors. Leptin is known to act as a mitogen, inflammatory and pro-angiogenic factor that induces cancer cell proliferation and tumor angiogenesis. Moreover, leptin signaling induces cancer stem cells, which are involved in cancer recurrence and drug resistance. A novel and complex signaling crosstalk between leptin, Notch and interleukin-1 (IL-1) [Notch, IL-1 and leptin crosstalk outcome (NILCO)] seems to be an important driver of leptin-induced oncogenic actions. Leptin and NILCO signaling mediate the activation of cancer stem cells that can affect drug resistance. Thus, leptin and NILCO signaling are key links between obesity and cancer progression. This review presents updated data suggesting that adiposity affects cancer incidence, progression, and response to treatment. Here we show data supporting the oncogenic role of leptin in breast, endometrial, and pancreatic cancers.

Key words: Obesity; Leptin; Breast cancer; Endometrial cancer; Pancreatic cancer; Notch, interleukin-1 and leptin crosstalk outcome

Core tip: Obesity is a global pandemic and a risk factor for a number of cancers. Obesity is characterized by high levels of body fat (adiposity) and leptin. Research shows that leptin and its oncogenic crosstalk Notch, interleukin-1 and leptin crosstalk outcome (NILCO) provide insight on the link between obesity and cancer progression. Thus, leptin and NILCO can act as mitogenic, inflammatory, and angiogenic cues promoting the progression of cancer, cancer stem cells, and drug resistance. This review shows updated information on leptin and NILCO's oncogenic roles in breast, endometrial, and pancreatic cancers.

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INTRODUCTION

Obesity is a global pandemic. As of 2014, there were at least 600 million obese adults across the globe[1]. The Centers for Disease Control and Prevention reports that 1/3 of the United States adult population, or 78.6 million, is obese[2]. The negative impact on the public health burden continues to increase with the number of overweight and obese individuals[3]. A quantitative meta-analysis of 33 United States studies lists the direct medical cost of obesity as $1723 per-person[4]. Annual medical costs incurred by the overweight and obese are still increasing throughout the United States. Many healthcare costs occur because obesity is a risk factor for several chronic diseases: Cardiovascular disease[5], type two diabetes mellitus[6], and several different types of cancer[7].

Clinical measures for obesity include having a body mass index (BMI) that is $\geqslant$ 30; clinical obesity can also be characterized by excessive accumulation of body fat[8]. BMI measures body fat using an individual's height to weight ratio[9]. Physicians, clinicians, nutritionists, and other scientists often assess an individual's BMI to determine obesity related health outcomes[10]. While BMI continues to be a common variable for overall assessment of obesity, it has become evident that BMI is not the only crucial factor when investigating how obesity works to drive the development or progression of other diseases[11]. Clinicians and researchers are now correlating body fat and waist circumference to increased risks for cardiovascular disease, type-two diabetes, and cancer[12-14]. The connection between obesity and cancer is complex and still unclear. However, research shows that adiposity is providing insight on the link between obesity and cancer progression[11].

Leptin and adiponectin are two key cytokines secreted from adipose tissue. Leptin levels are often chronically elevated in human obesity[15]. Chronically high levels of leptin in the overweight and obese can lead to a mechanism known as "leptin resistance"[16]. Leptin resistance can lead to loss of appetite control, increased food intake and accumulation of fat in adipose tissue[17]. These issues could be crucial during cancer development as leptin mediates several signaling pathways that are essential to angiogenesis, cell proliferation, migration, and survival[18].

Leptin has been shown to play a role in several types of cancers. The expression of leptin and its receptor (OB-R) has been reported in many cancer types including: Gliomas, Carcinomas, Adenocarcinomas, and Melanomas[18]. Obesity signals (leptin) have been linked to the progression of several cancers. The connection between obesity signals (leptin and/or OB-R) and cancer progression has been detected in bladder, brain, breast, colon, endometriod, esophageal, kidney, liver, lung, ovarian, prostate, skin, and thyroid cancers[18].

This review shows updated information on leptin's oncogenic role in breast, endometrial, and pancreatic cancers. We present experimental data obtained by using different research methodologies [including: Cell culture, animal trials, flow cytometry, immunological methods, polymerase chain reaction (PCR), *etc.*] suggesting that adiposity affects cancer progression. The effect of adiposity on cancer progression, leptin signaling mediated activation of cancer stem cells, and the link between leptin and drug resistance is discussed.

LEPTIN STRUCTURE, SOURCE, AND FUNCTION

Discovered in 1994[19], leptin is a 16 kDa protein hormone that is composed of 167 amino acids and is coded by the *LEP* gene (also known as the *OB* gene)[18]. This small protein binds to the leptin receptor (OB-R) leading to control of leptin ligand/receptor mediated pathways. Leptin binding to OB-R is highly specific. Indeed, leptin only binds OB-R, and this receptor can only binds leptin. OB-R belongs to the class I cytokine receptor superfamily composed by six different isoforms produced *via* alternative mRNA splicing. The long form OB-Rl or OB-Rb, with full signaling capabilities, carries out primary biological functions of leptin, while short OB-R isoforms induce secondary signaling pathways[18,20].

Through canonical leptin signaling, the secreted protein enacts its hormonal potential to control appetite, energy balance, and glucose homeostasis *via* negative feedback[21]. When energy levels are high, meaning the body has high triglyceride (fat) stores, the hypothalamus sends signals received by receptors of adipocytes[16]. Adipocytes produce and secrete leptin and the protein then circulates to the brain *via* endocrine signaling[21]. Leptin then binds the extracellular domain of the long form OB-R that activates an associated

JAK2 protein[18,22,23]. JAK2 phosphorylates three tyrosine residues (Tyr985, Tyr1077 and Tyr1138) on the intracellular portion of the OB-R[18,23,24]. Phosphorylation and activation of these three residues in hypothalamic cells initiates the downstream signaling that ultimately regulates the negative feedback responsible for establishing satiety, maintaining energy/glucose balance, and regulation of reproduction[24]. However, these mechanisms become dysfunctional during obesity as leptin continue to rise until the body no longer responds to endogenous leptin signaling pathways[25]. This phenomenon is known as leptin resistance. Obese individuals are often leptin resistant. During leptin resistance circulating leptin levels increase in the body, however, there is a breakdown in the mechanism that signals satiety resulting in the accumulation of excess leptin. High leptin levels can induce cancer cell proliferation and, therefore, could be a key link between obesity and cancer progression[26].

LEPTIN AND OB-R EXPRESSION IN BREAST CANCER

Understanding the role of leptin in normal breast development is important when describing how leptin and OB-R could affect breast cancer progression. Human breast are primarily composed of adipose tissue, thus this organ is a major site of leptin production and secretion. It has been demonstrated that leptin signaling plays a role in the development of mammary glands[27]. The importance of leptin and OB-R expression has been tested in murine models. In one study, it was demonstrated that mutant mice with deficient leptin signaling (either lacking leptin *ob/ob*, or lacking functional OB-R *db/db*) show abnormal mammary glands[27]. Additionally, *ob/ob* and *db/db* mice show very low incidence of mammary tumors[27]. However, it is important to note that normal human mammary gland tissue show low OB-R expression. Conversely, cancerous cells found in the mammary gland overexpress OB-R and respond to leptin stimulus by increasing production of angiogenic factors [vascular endothelial growth factor (VEGF), VEGF receptor 2 (VEGFR-2)], increasing proliferation, and survival[26,28,29]. These findings suggest that leptin signaling mediates proliferative pathways in normal and malignant breast cells.

Leptin oncogenic effects on breast cancer

Leptin has been shown to increase proliferation of estrogen responsive (ER^+) and unresponsive (ER^-) breast cancer cells *in vitro*[30]. Moreover, inhibition of leptin signaling has been show to abrogate ER^+ and ER^- breast cancer growth[31]. Recently, immunohistochemical analysis of breast cancer tissue samples obtained from Chinese patients (n = 67) confirmed a strong correlation between leptin expression and breast cancer progression, as 61% of the samples were positive for leptin and OB-R[26,32,33]. Studies continue to be published highlighting leptin's effects on proliferative pathways in breast cancer.

Leptin mediated induction of Cyclin D1 and Cdk2 has been shown in breast cancer cells *in vitro*[30,34]. This was further assessed by Zheng *et al*[35] who have confirmed that Cyclin D1 expression is regulated by active leptin signaling in the MMTV-Wnt-1 transgenic mouse[35]. We recently showed that inhibition of leptin signaling in triple negative breast cancer cells (TNBC) [using an innovative leptin antagonist bound to nanoparticles (IONP-LPrA2)] abrogated leptin-induced S phase progression of the cell cycle[36]. These data suggest that leptin is essential in promoting S-phase cell cycle progression in breast cancer.

The effects of leptin signaling on proliferative pathways have also been linked to telomerase activity. Leptin has been shown to induce telomerase activity in MCF-7 breast cancer cells in a dose-dependent manner. Moreover, leptin signaling may be a key promoter of senescence evasion in human breast cancer cells *via* up regulation of telomerase activation[37]. These authors also found that leptin activates the transcription of hTERT (the enzyme responsible for reverse transcriptase of telomerase). Taken together those findings suggest that leptin signaling increases cell proliferation *via* telomerase activation in breast cancer.

Leptin can also increase survival of breast cancer cells *via* additional mechanisms. Apoptosis evasion mechanisms in human breast cancer allows for the growth of solid tumors. Increase survival of TNBC *via* leptin signaling was demonstrated by Ray *et al*[38]. These authors found an inverse relationship between the expression of apoptotic protein in TNBC treated with leptin[38]. Leptin induced Bcl-xL and Bcl-2 protein levels and increased survival of TNBC[38]. These survival mechanisms are punctuated by apoptosis evasion *via* leptin's ability to regulate Bcl-2 proteins in human breast cancer cells[38].

The expression of VEGF and its receptor, VEGFR-2, is instrumental for the formation and function of vasculature in tissues. VEGF binding to VEGFR-2 leads to signaling cascades that result in neovascularization[39]. Additionally, VEGF/VEGFR-2 autocrine and paracrine actions in breast cancer have been shown to play an important role in cancer cell survival[28].

Leptin is a non-classical pro-angiogenic factor that has an essential role in tumor angiogenesis[40]. We have previously shown that leptin induces breast cancer growth and significantly increases production of both VEGF and VEGFR-2 supporting tumor angiogenesis[28,29,31]. The IL-1 system has also been linked to angiogenesis *via* leptin's ability to upregulate VEGF/VEGFR-2 in breast cancer[41]. Direct leptin induction of IL-1 can indirectly upregulate VEGF/VEGFR-2[41].

Novel molecular links between inflammatory and angiogenic responses of leptin-stimulated human endothelial cells (hECs) were previously demonstrated. hECs were also shown to be a target of leptin signaling through the transactivation of VEGFR-2's intracytoplas-

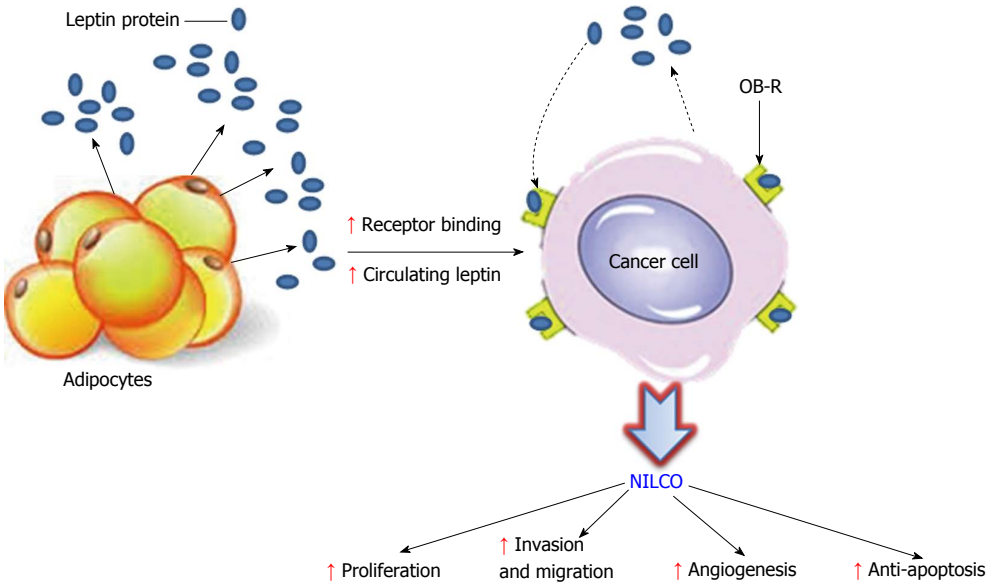

Figure 1 Obesity, leptin, Notch, interleukin-1 and leptin crosstalk outcome, and cancer progression. The complex relationship between obesity, leptin and Notch, IL-1 and leptin crosstalk outcome (NILCO) that occurs in cancer cells is illustrated here. Leptin secreted by adipocytes or cancer cells binds to its receptor, OB-R, on cancer cells. Leptin signaling crosstalks with Notch and IL-1 systems to induce survival, proliferation, invasion, migration, angiogenesis and anti-apoptosis of cancer cells. These leptin actions have also been shown to lead to the progression of breast, endometrial and pancreatic cancer[29,71,85,89]. OB-R: Leptin receptor; IL-1: Interleukin-1.

matic tail and upregulation of enzymes involved in inflammatory pathways. In cultured human umbilical vein endothelial cells (HUVEC) leptin stimulated rapid phosphorylation of VEGFR-2 on Tyr (1175) and increased cyclo-oxygenase-2 (COX-2) expression *via* p38 mitogen-activated protein kinase (p38 MAPK) and Akt. Moreover, inhibition of these leptin-induced pathways and leptin/OB-R signaling (*via* the peptide LPrA2, a leptin antagonist produced by us) abrogated leptin-induced capillary-like tube formation by HUVEC on Matrigel. A functional endothelial p38(MAPK)/Akt/COX-2 signaling axis triggered by leptin/OB-R-induced VEGFR-2 transactivation is required for leptin's pro-angiogenic actions in hECs[42]. More recently we have shown that leptin also induced phosphorylation of VEGFR-2 at sites Y951, Y996, Y1059 and Y1175 in porcine aortic ECs overexpressing VEGFR-2. Protein expression of Notch4 and Jagged1 was also induced by leptin treatment in fibroblast cells (NIH/3t3). Therefore, leptin secreted by fibroblast cells and/or adipose tissue may contribute to tumor angiogenesis by acting directly on stromal cells and inducing a VEGFR-2/Notch crosstalk[43].

Proliferation and acquisition of malignant features in breast cancer cells has illustrated the important role of leptin signaling and crosstalk between several cellular pathways[44]. A leptin-induced complex crosstalk between several factors [Notch, IL-1 and leptin crosstalk outcome (NILCO)] was detected in breast cancer cells and has been shown to drive cell survival and tumorigenesis[44] (Figure 1). NILCO is an advanced model that provides evidence, and works to explain, the crosstalk outcomes that occur as a result of leptin signaling between Notch family proteins and IL-1 inflammatory systems. NILCO could represent the integration of developmental, pro-inflammatory and pro-angiogenic signals critical for leptin-induced cell proliferation/migration and regulation of VEGF/VEGFR-2 in breast cancer[44].

Notch is a hallmark of breast cancer[44]. Notch is a family of transmembrane proteins that act as receptors of specific ligands expressed in the membrane of adjacent cells. Notch signaling activation is mediated by binding to those ligands followed by a series of proteolytic events of Notch intracytoplasmatic tail *via* ADAM protease and γ-secretase, which have been shown to regulate cell differentiation[45]. Notably, our group has recently shown that leptin induces Notch activation in estrogen responsive and TNBC cells *in vitro* and *in vivo*[46]. Moreover, we have shown that leptin induced cell proliferation and migration are Notch dependent[46]. To investigate whether obesity induces a leptin-Notch signaling axis in breast cancer, Notch was determined in human MCF-7 and MDA-MB231, and mouse E0771 cells and in E0771-tumors hosted by syngeneic lean and diet-induced obesity C57BL/6J female mice. Notch loss-of-function [*via* inhibition of γ-secretase with DAPT and transfection of dominant negative (R218H) RBP-Jk (CSL/CBF1)] showed that a functional leptin-Notch signaling axis was involved in the proliferation and migration of E0771 cells. These data suggest that leptin induced Notch could be involved in the reported higher incidence, aggressiveness, and poor prognosis of breast cancer in obese patients[44,46].

NILCO biomarkers were found differentially expressed in ER^+, ER^- and TNBC tissues obtained from Chinese women. TNBC showed differential localization patterns of NILCO. TNBC showed fewer nuclei and cytoplasms positive for Notch4 and JAG1, but more cytoplasms were positive for leptin. Additionally, fewer

TNBC stromas were positive for Notch1 and Notch4, but 100% of TNBC stromas were positive for VEGFR-2. Moreover, TNBC had lower DLL4 and IL-1R tI expression. Remarkably, analysis of NILCO and targets using Pathway Studio9 software (Ariadine Genomics) showed multiple molecular relationships that suggest NILCO has potential prognostic biomarker value in breast cancer[32].

Our lab has also shown that leptin-induced Notch and IL-1 inflammatory systems are involved in the regulation of breast cancer cell survival and proliferation. Concurrent activation of NILCO leptin signaling has been shown to be instrumental in the proliferation of breast cancer cells during spontaneous mammary tumor formation in obese mice that are resistant DMBA [7, 12-dimethylbenz(a)anthracene]-induced cancer[47].

Taken together these mechanisms work to increase angiogenesis, cell proliferation, and survival in breast cancer that could be of utmost relevance for obese patients (Figure 1). Targeting NILCO may help to design new pharmacological strategies aimed at controlling breast cancer growth and angiogenesis[18].

Leptin, epithelial to mesenchymal transition and tumor stroma

Leptin signaling induces adhesion, increases migration, and invasion in breast cancer cells *in vitro*[44]. Cancer cells that gain an increased ability to migrate and invade surround tissues have undergone an epithelial to mesenchymal transition (EMT). It was found earlier that leptin inhibition decreased EMT and migration of hECs *via* signal transducer and activator of transcription 3 (STAT3) and Snail/vascular endothelial cadherin-independent mechanism. Moreover, leptin signaling in ECs was related to Akt signaling pathway, αvβ3 integrin receptor and matrix metalloprotease 2, suggesting that leptin induces adhesion and migration processes[48]. Furthermore, leptin was shown to stimulate phosphorylation of glycogen synthase kinase 3β (GSK3β) *via* Akt activation that decreased GSK3β-LKB1-Axin complex formation and induced β-catenin, Wnt1 and MTA1 expression. Moreover, leptin-treated breast tumors hosted by mice showed increased expression of Wnt1, pGSK3β, β-catenin and vimentin, but reduced E-cadherin expression. A novel crosstalk between leptin and MTA1/Wnt signaling was found in breast cancer cell-EMT[49].

Research associated with obesity, leptin, and breast cancer progression continues to become more complex. Studies that focus on the tumor microenvironment are currently providing a framework for the overlap between several leptin mediated pathways. Increasing amounts of research show that several cell components of the breast cancer stroma (*i.e.,* cancer associated fibroblasts, macrophages, adipocytes, and cancer stem cells) are influenced by leptin signaling[44,50]. Moreover, emerging data also show that tumor stroma cells secrete molecules that bolster survival for breast cancer cells. During this process, adipocytes secrete many factors, including leptin and the inflammatory cytokine IL-6, activating paracrine signaling that leads to action of the JAK2/STAT3 pathway[51]. The end result is an activation of pathways that confer stem-cell-like properties ($OCT\text{-}4^+$/$SOX2^+$) to the breast cancer cells[51]. Our group has identified numerous other cytokines and inflammatory factors (Notch1-4, IL-1, VEGF and VEGFR-2) that are now shown to bolster breast cancer progression *via* the tumor microenvironment[41,44]. We, and others, have also found that leptin can crosstalk with many oncogenic signals and induce secretion of chemotaxis factors for macrophages in the mammary gland environment eliciting pro-inflammatory changes that lead to malignant transformation of cells[18,52,53].

It was proposed that obesity impacts breast cancer not only systemically but also at the local level in the breast. Paracrine factors resulting from the crosstalk among adipocytes, tumor cells and macrophages in the breast tumor microenvironment might contribute to tumor progression *via* additional mechanisms. Indeed, breast adipose tissue enables the crosstalk between adipocytes and breast tumor cells contributing to tumor macrophage recruitment. *In vivo* experiments demonstrated that mammary tumors from obese mice are larger, and that their associated adipose tissue contained higher numbers of activated macrophages and hypertrophic and more inflamed adipocytes. Then, breast adipose tissue could play an additional role in breast cancer development in obesity by recruiting and activating macrophages[52].

Leptin, obesity, and breast cancer therapies

The Food and Drug Administration authorized the use of Tamoxifen (TAM), a selective estrogen receptor modulator, for treatment of breast cancer in 1998. TAM is widely used as the first line drug for chemoprevention of pre-menopausal/post-menopausal women at high risk of breast cancer. TAM targets ER^+ and/or progesterone responsive (PR^+) breast cancers[54]. Additionally, TAM is indicated for reduction of contra lateral BC risk[55]. Therefore, the National Cancer Institute recommends long-term TAM chemoprevention. To date more than 7 million patients a year use TAM[56].

Traditional therapeutic breast cancer treatments can often become taxing on patients due to several adverse side effects (fatigue, pain, loss of appetite, nausea, vomiting). Additionally, cancer cell populations often become resistant to therapies[39,57]. Breast cancer patients treated with TAM often show drug resistance, causes for this are not completely known. However, it could be linked to obesity signals (*i.e.,* leptin). TAM induces an increase in leptin expression[58]. In turn, leptin can transactivate ER, and increase aromatase activity, which leads to the induction of estrogen synthesis[59]. Reciprocally, estrogen signaling can induce leptin and OB-R expression, increase the development of vascular thrombosis[60] and impair TAM effects[58].

It was shown that leptin counters the chemotherapeutic actions of TAM in breast cancer cells *in*

vitro[61]. Moreover, TAM-resistant breast cancer cells were less proliferative *in vitro* when OB-R was knocked-down[57]. Consequently, leptin signaling has become a target for the development of new inhibitors that can be used as adjuvants during chemotherapeutic treatments[33,62].

A recent finding has reported the role the synergistic relationship between leptin and STAT3 phosphorylation as a mediator of TAM resistance in breast cancer cells[63]. When treated *in vitro* with 2 μmol/L of TAM for 72 h, ER positive MCF-7 and MCF-7/HER2 cell lines showed a statistically significant decrease in cell viability as measured by MTT assay[63]. However, 72 h combination treatment of MCF-7 and MCF-7/HER2 cell lines with 2 μmol/L TAM + 200 ng/mL leptin had a restorative effect on cell viability[63]. The study used Western blot analysis of p-STAT3, OB-R, HER2, and ER to investigate leptin's role in STAT3 phosphorylation in the presence of TAM. STAT3 phosphorylation (activation) increased in MCF-7 cells treated with TAM alone and TAM plus leptin[63]. In contrast, MCF-7/HER2 cells treated with TAM alone had decreased expression of phosphorylated STAT3[63]. More interestingly, leptin restored phosphorylated STAT3 to levels that were comparable to untreated cells in the presence of TAM at 24 h and 7 d time points[63]. This research provided insight on two key mechanistic pathways that could be critical for decreasing TAM resistance in obese breast cancer patients.

However, recent studies investigating the effects of TAM treatment in obese and non-obese ER^+ breast cancer patients are showing that TAM may continue to be an effective treatment for obese patients suffering from the disease. Analysis of the data acquired after the National Surgical Adjuvant Breast and Bowel Project protocol B-14, shows that overall mortality rates were reduced in both obese and non-obese women who were treated with TAM when compared to women with similar BMI who received a placebo[64]. Obese patients (n = 687) had higher TAM/placebo hazard ratios for breast cancer recurrence, contralateral breast cancer, total mortality, and mortality after breast cancer events compared to underweight (n = 83), normal weight (n = 1593), and overweight (n = 1022) patients[64]. However, TAM effectively reduced breast cancer recurrence and mortality rates across all BMIs, and there was no statistically significant increase in mortality for obese women[64].

A published secondary analysis of the double blind Arimidex, Tamoxifen Alone or in Combination clinical trial shows that overall recurrence rates were equal in ER^+ breast cancer patients who were treated with TAM[65]. Similarly, the Austrian Breast and Colorectal Cancer Study Group trail 6 reports that there was no difference in outcomes (disease-free survival, distant recurrence-free survival, and overall survival) between obese and non-obese women who received TAM treatment[66]. However, obese women who received TAM in combination with an aromatase inhibitor had worse outcomes than non-obese counterparts receiving the same treatment[66]. These studies show that there may be strong associations between body weight and TAM efficacy. Azrad *et al*[67] have presented additional background on this subject including studies that compare the efficacy of TAM and aromatase inhibitors. A major section of Azrad's review provides data from four clinical studies and each suggests that aromatase inhibitors are less effective than TAM when treating women with hormone-receptor positive breast cancers[67].

Links between leptin levels and chemotherapeutic resistance mechanisms have also been identified in the MCF-7 breast cancer cell line *in vitro*[68]. MCF-7 cells were treated with 100 ng/mL of leptin for 10 d, on the 9^{th} day cells were treated with 10 μmol/L concentration of cisplatin (CIS)[68]. The effect of chronic leptin exposure on cell proliferation during CIS treatment was measured using Crystal Violet assays[68]. The data included in the study showed that chronically high leptin concentrations counteracted CIS-induced cytotoxicity in MCF-7 breast cancer cell line *in vitro*, which supports the notion that leptin works as a survival factor that confers chemotherapeutic resistance in breast cancer[68].

TNBC are refractory to hormonal therapies, do not have a targeted therapy and are mainly treated with chemotherapeutic drugs. TNBC patients often develop drug resistance. The mechanisms leading to chemotherapy resistance in TNBC patients are still unclear. Data show that obesity may lead to chemo-resistance in breast cancer. Increasing BMI of TNBC patients was associated with significantly more advanced disease and higher incidence and lower response rates to chemotherapeutics[69].

Breast cancer stem cells (BCSC), population of drug-refractory cancer cells with self-renewal capabilities, have been linked to the development of drug resistance and are present in TNBC cell lines and derived tumors[70]. Importantly, leptin activates several molecules critically associated with BCSC, (*i.e.,* Notch, Akt, STAT3, nuclear factor-κB)[71-73]. Our preliminary data further suggest that leptin induced stemness and drug resistance in breast cancer cells. Leptin increased the levels of several genes and molecules associated with BCSC maintenance, and cellular markers CD44 and ALDH1. Notably, OB-R expression and STAT3 phosphorylation (leptin's main downstream effector) are characteristic features of tumor and embryonic stemness, which are mediated by a feedback mechanism involving the pluripotency-associated transcription factors, OCT4, SOX2 and NANOG[72]. We further tested the effects of leptin in human ER^+ (MCF-7) and TNBC cell lines (HCC1806) exposed to various concentrations of CIS[74], sunitinib, paclitaxel, and doxorubicin[36]. Leptin induced a significant increase in cell survival that was abrogated by the use of a leptin peptide receptor antagonist-conjugated to iron oxide nanoparticles (IONP-LPrA2)[36]. Therefore, the inhibition of leptin signaling could help to the reduction of drug resistance and increase effectiveness of chemo-

therapeutic drugs used for BC, especially in obese contexts.

ENDOMETRIAL CANCER

Endometrial cancer (EmCa) is the most frequent gynecological malignancy of the female reproductive tract, and is the fourth most commonly diagnosed new cancer among women in the United States following breast, lung/bronchus, and colorectal cancers[75,76]. The 2015 Surveillance, Epidemiology, and End Results Program report from the National Cancer Institute (NCI) estimates that 54870 women will be diagnosed with EmCA in the United States, and roughly 10170 women will succumb to the disease. The 5-year survival rate is favorable at 96% when diagnosed at a local stage and decreases to 16% when it is diagnosed at distant sites[77].

EmCa can be classified as type Ⅰ and type Ⅱ[75]. Type Ⅰ EmCa accounts for 85% of all EmCa cases, and is thought to be caused by un-opposed estrogen stimulation, and is considered low grade with a favorable prognosis[75]. Indeed, type Ⅰ EmCa is less aggressive and less likely to spread to distant sites[75,78]. Type Ⅱ makes up approximately 10% of EmCa, but its etiology is still unclear, and seems to be independent from estrogen stimulation. Type Ⅱ EmCa shows high grade, low differentiation, and poor prognosis[75]. Moreover, type Ⅱ EmCa is more likely to metastasize to distant sites[75,78]. Type Ⅱ EmCa includes several subtypes (*i.e.,* papillary serous carcinoma and clear-cell carcinoma)[75]. Less frequent EmCa types, malignant mixed mullerian tumors or carcinosarcomas, are considered Type Ⅱ tumors that represent approximately 4% of uterine cancers[78].

The incidence of EmCa is highest in Caucasian women (24.8/100000) when compared to African American women (21.8/100000), and other ethnic groups[77]. However, African American women are more likely to die from EmCa when compared to Caucasian women. Mortality rates in African American women (7.3/100000) are higher than in Caucasian women (3.9/100000)[77]. The basis for this health disparity is ambiguous, but could be due to factors which include but are not limited to socioeconomic status, lack of access to healthcare, type of healthcare provided, culture, lifestyle, and/or biological differences between patients belonging to diverse ethnic groups[77].

Obesity and EmCa incidence strongly correlate[7]. Approximately 40% of EmCa cases are related to obesity[75]. EmCa is more than three times as common in obese women when compared to normal healthy weight women[75]. The relationship between obesity and EmCa incidence and progression is characterized by elevated levels of estrogens (unopposed estrogen stimulus), insulin growth factor-1, adipokines (leptin; resistin), and cytokines[75]. Clinical data have been published showing links between obesity, EmCa type, and race. The study showed that 55.3% (n = 871) of the women diagnosed with type Ⅰ EmCa were obese, while 36% (n = 64) were obese and more likely to belong to a non-white race[79]. Although this study did not clearly define the relationship between obesity and type Ⅱ, the data suggest that obesity and health disparities play a role in EmCa.

Leptin signaling was correlated with the expression of several pro-angiogenic factors in EmCa cell lines[75]. In An3Ca, Ishikawa, and SK-UT2 (malignant endometrial epithelial) cell lines leptin regulates VEGF, IL-1β, LIF and their respective receptors. However, IL-1β was only increased by leptin in benign primary endometrial cells. IL-6, resistin, and tumor necrosis factor α are additional factors involved in leptin oncogenic crosstalk in EmCa[18]. The short OB-R isoforms are expressed higher than the long OB-R isoform in EmCa[75,80].

As previously described in this review, NILCO, a complex crosstalk between leptin and pro-angiogenic, inflammatory and mitogenic factors occurs in breast cancer[44]. Additionally, NILCO could also be present in EmCa[75]. Our preliminary data suggest that leptin signaling and NILCO may be associated with the more aggressive type Ⅱ EmCa, which affects more postmenopausal and African-American women. Studies using type Ⅱ EmCa tissue microarrays from Chinese and African American patients assessed this notion[75].

We have previously reported an interesting observation showing that NILCO components are differentially expressed in type Ⅰ and type Ⅱ EmCa[75]. Table 1 shows the expression levels of NILCO components in type Ⅰ and type Ⅱ EmCa from obese African American women and lean Chinese women. Immunohistochemical staining, Western Blot and Real-time PCR analyses confirm that NILCO was expressed higher in type Ⅱ EmCa. These data suggested that the more aggressive and non-hormonal type Ⅱ form of EmCa may be dependent on Notch signaling. The results may also suggest that an active crosstalk between obesity related leptin signals and Notch occurs in EmCa. Therefore, NILCO expression in EmCa may serve as a new tumor marker.

Androgens, estrogens and leptin in the menstrual cycle

Estrogens are the main regulators of the menstrual cycle. Estrogens are mainly produced by the ovaries and regulated by neuroendocrine hormone signaling[81]. However, estrogens are also synthesized by adipose tissue[82]. In fact, estradiol levels varied throughout the menstrual cycle between women with different body fat content[83]. Women with both very low and very high body fat had significantly lower estradiol levels during the follicular phase and midcycle during their menstrual cycle[83].

Androgens are produced and accumulated in adipose tissue. They can be converted into estrogen *via* the actions of aromatase. Excessive size of adipose tissue can convert androgens into estradiol and estrone *via* aromatase providing an important estrogenic surge in obese patients[75]. Therefore, these molecules could alter female reproductive function and hormonal equilibrium especially after menopause in obese women[84]. Andro-

Table 1 Expression of Notch, interleukin-1 and leptin crosstalk outcome components in African American and Chinese women suffering from endometrial cancer

	Endometrial cancer					
	African American women			Chinese women		
	Type Ⅰ (n = 12)	Type Ⅱ (n = 17)		Type Ⅰ (n = 97)	Type Ⅱ (n = 23)	
NILCO IHC	H SCORE	H SCORE	*P* value	H SCORE	H SCORE	*P* value
Notch1	1.19	1.80	< 0.01	1.00	1.78	< 0.01
Notch2	1.10	1.30	0.05	1.00	1.15	> 0.05
Notch3	1.15	1.45	> 0.05	1.10	1.20	> 0.05
Notch4	1.50	1.96	< 0.01	1.10	1.58	< 0.05
JAG1	1.36	2.20	< 0.01	1.30	1.87	< 0.01
DLL4	1.80	2.49	< 0.01	1.31	1.80	< 0.01
Survivin	1.20	1.96	< 0.01	1.17	1.60	< 0.01
OB-R	1.60	1.73	< 0.01	1.10	1.50	< 0.05
IL-1R t Ⅰ	1.28	2.00	< 0.01	1.40	1.73	< 0.05
Hey2	1.14	1.45	< 0.01	1.07	1.30	< 0.05
WB	Protein expression	Protein expression	*P* value			
Notch1	48	58	< 0.05			
Notch2	38	36	> 0.05			
Notch3	48	44	> 0.05			
Notch4	44	98	< 0.01			
JAG1	140	172	< 0.05			
DLL4	40	115	< 0.01			
Survivin	131	230	< 0.05			
OB-R	25	70	< 0.01			
IL-1R t Ⅰ	59	109	< 0.05			
Hey2	46	100	< 0.01			
qPCR	mRNA expression	mRNA expression	*P* value			
Notch1	1.00	1.30	< 0.01			
Notch3	0.45	0.80	< 0.05			
Notch4	0.80	1.40	< 0.01			
JAG1	0.05	0.52	< 0.01			
DLL4	1.10	1.50	< 0.01			
Survivin	0.48	0.51	< 0.05			
OB-R	0.45	0.65	> 0.05			
IL-1R t Ⅰ	0.82	1.56	< 0.01			
Hey2	0.03	0.62	< 0.01			

NILCO: Notch, interleukin-1 and leptin crosstalk outcome; IHC: immunohistochemistry; H SCORE[32]: Semi-quantitative value calculated for each antigen as determined by the following equation H SCORE = $\sum$pi(i + 1); WB: Western blot; qPCR: Real-time polymerase chain reaction; Notch1-4: Transmembrane receptors; JAG1: Jagged 1; DLL4: Delta like-4 protein, and Notch ligand; Survivin: An anti-apoptotic factor and Notch target; OB-R: Leptin receptor; IL-1R t Ⅰ: Interleukin 1 receptor type Ⅰ; Hey2: Hes-related family BHLH transcription factor with YRPW motif 2 and Notch ligand.

gens and estrogens influence the menstrual cycle. In normal weight women, testosterone fluctuates throughout the menstrual cycle and peaks during the ovulation phase[84]. Conversely, androstenedione and dehydroepiandrosterone showed no significant variations throughout the menstrual cycle[84]. Androstenedione levels were found to peak at ovulation[85]. Yet, epidemiological studies have shown increased EmCa risk among pre- and postmenopausal women who have elevated plasma androstenedione and testosterone, and among postmenopausal women who have increased levels of estrone and estradiol. Interestingly, free testosterone levels were significantly higher in obese women when compared to non-obese women and slight variations of testosterone were observed during each phase of the menstrual cycle[86].

Also, the menstrual cycle may be influenced by the levels of serum leptin[87]. In obese women, the highest serum leptin levels are observed during the luteal phase. Similarly, an increase in estradiol levels coincided with the increase in serum leptin levels[87]. However, serum leptin levels were unchanged throughout the menstrual cycle of women with normal weight[87].

PANCREATIC CANCER

Pancreatic cancer (PC), including pancreatic adenocarcinoma, has been the fourth leading cause of cancer related death in the past few decades. The risk factors associated with PC are chronic pancreatitis, diabetes, smoking and high BMI (> 30). Obesity, pandemic in the United States, has been linked to PC: Increased BMI was associated with more advanced stage at diagnosis; 72.5% of obese patients presenting metastatic disease compared to 59.4% of the normal weight patients[88]. A positive significant association between waist circumference and PC was determined in a combined meta-analysis of cohort studies for the Asia-Pacific region[89].

There are few reports on the role of leptin in PC. It was shown that high levels of leptin were associated

Table 2 Leptin signaling impacts on pancreatic cancer progression

Treatments	Human pancreatic cancer cell lines										
	BxPC-3 less aggressive				MiaPaCa-2 more aggressive		Panc-1 more aggressive				
	Control	Leptin	Leptin + CT	Leptin + CT + LI	Control	Leptin	Control	Leptin	CT	Leptin + CT	Leptin + CT + LI
Proliferation (%)	100	212[2]			100	120[2]	100	130[2]			
Survival[1] (%)	100	100	63[2]	55[2]			100	100	35[2]	41[2]	36[2]
PCSC (%)	100	130-140			100	120-130					
Notch1	100	170[2]			100	NC					
Notch2	100	147[2]			100	75					
Notch3 (%)	100	NC			100	NC					
Notch4	100	71			100	146[2]					
Tumorsphere formation (%)	100	197[2]			100	184[2]	100	221[2]			

[1]Cell survival was determined by flow cytometry and MTT assays in media containing 10% fetal bovine serum (BxPC-3) or serum free medium containing leptin (Panc-1); [2]Statistically significant ($P < 0.05$) *vs* control. CT: Chemotherapy, LI: Leptin inhibitor 90; PCSC: Pancreatic cancer stem cell markers; NC: No change.

with PC development. In a pooled analysis from PC patients, it was found that an association between leptin levels and elevated OB-R expression in PC correlated to the stem cell marker OCT-4[90]. Overexpression of leptin was shown to significantly promote the growth of human PC xenografts and lymph node metastasis in mice[91]. We have found that leptin significantly increased Notch1 and Notch2 expression in BxPC-3 cells, and Notch4 in MiaPaCa-2 cells. Therefore, Notch induced by leptin could be involved in PC progression, and could be a link between obesity and PC.

There are some reports[92,93], showing that leptin inhibited proliferation of PC cells *in vitro*. However, in our recent studies using PC lines that showed different degrees of aggressiveness: BxPC-3 (less aggressive) and MiaPaCa-2 and Panc-1 (more aggressive), we found that leptin signaling increases PC cell progression *in vitro* (Table 2)[94]. Leptin-induced cell proliferation was determined by MTT and cell cycle assays. PC cells treated with leptin increased proliferation, survival, and expression of stem cells markers [PC stem cell (PCSC)], and the ability to produce tumorspheres *in vitro*, which are features that characterize enhanced tumorigenesis (Table 2)[88].

Present data further support the notion that leptin can accelerate PC growth. The reason for these discordant data is unknown.

Leptin signaling has also been suggested to influence PCSC populations. Increased expression of $CD24^+/CD44^+$ markers in PC correlated to higher tumorsphere formation. Moreover, triple positive PC cells ($CD24^+/CD44^+$/ESA) were identified as PCSC[95]. Our preliminary data show that leptin significantly increased PCSC ($CD24^+/CD44^+$/ESA) and tumorigenesis (formation of tumorspheres) *in vitro* in both BxPC-3 and MiaPaCa-2 cells. PCSC are believed to play a role in drug resistance. Indeed, leptin-induced survival of PC cells treated with chemotherapeutics was abrogated by the addition of IONP-LPrA2, a specific leptin signaling inhibitor coupled to iron oxide nanoparticles. Therefore, the inhibition of leptin signaling *via* IONP-LPrA2 might be used as an adjuvant therapy with current chemotherapeutic drugs, and could lead to a new way for prolonging survival of obese PC patients.

EFFECTS OF WEIGHT LOSS ON LEPTIN LEVELS IN CANCER

Despite the recognized role of overweight and obesity on cancer incidence, the majority of the clinical trials addressing BMI reduction are relegated to breast cancer. Few reports are available for endometrial and pancreatic cancers. A retrospective study suggests bariatric surgery may improve quality of life for morbidly obese women suffering from low risk type I endometrial cancer[96]. Clinical insights on the effects of weight loss in endometrial cancer patients include a retrospective study with findings that suggest weight loss after diagnosis and treatment may lead to poor prognosis[97]. Similar findings on the deleterious effects of weight reduction in pancreatic cancer patients show that significant weight loss during or following treatment correlates to poor post-treatment outcomes[98,99].

It is believed that obesity may promote the progression of ER^+ breast cancer in post-menopausal obese women *via* increased production of the estrogens by adipocytes[100]. In contrast to endometrial and pancreatic cancer data, long-term survival prognosis after treatment is poorer in overweight or obese who suffer from pre or post-menopausal

breast cancer[101]. Several clinical studies show trends between weight loss and lower leptin levels in patients with breast cancer. One recent study, randomized patients into low fat (n = 73) or low carbohydrate (n = 66) diet intervention groups to determine how weight loss affects plasma leptin and adiponectin levels in overweight or obese postmenopausal breast cancer survivors[102]. Following the 6 mo diet intervention the women in both groups, exhibited significant reductions in body weight and fat mass. Results from this trial show that the mean leptin level of the patients prior to intervention was 36 ng/mL, more than a 3 fold increase to concentration associated with normal weight (5-10 ng/mL)[102]. Interestingly, 50% of the patients had circulating adiponectin levels that are the same as normal weight women[102]. Moreover, the diet interventions decreased leptin levels by 92% of the patients; yet only 32% of the patients showed a decrease in adiponectin levels during intervention. However, this study did not address the impact of weight loss and adipokine reduction on breast cancer recurrence. Knowing how reduction of body weight and leptin levels affects breast cancer progression would be instrumental for the design of new chemotherapeutics. Obtaining clinical information on the mechanisms linking body weight loss, cancer progression, and recurrence could be also be key for developing preventative strategies that target leptin mediated breast cancer progression in obese women.

CONCLUSION

To date, several clinical trials have aimed to reduce body weight in cancer patients and have successfully implemented intervention strategies that have led to sustained weight loss in patients who have been treated for different types of cancer[96,102,103]. Consequently, many of these clinical studies predict that weight loss may be a major mediator of cancer progression, and majority of such studies focus on breast cancer[101-104]. Disparagingly, there currently are not many trials that report overall survival and/or cancer recurrence rates in obese patients who have successfully sustained weight loss after cancer treatment. Thus, data reporting how weight loss may improve outcomes in cancer patients have not been well documented. One reason explaining the lack of clinical data in this area may be that funding for large scale trials is not readily available[104]. An additional factor may be that the number of patients who are motivated and willing to participate in such studies is insufficient.

Leptin levels correlated to adiposity, and are elevated in obese individuals. Then, body weight loss decreases leptin levels. Leptin signaling is a key player in the progression of several types of cancer. Increasing evidences continues to implicate the role of leptin signaling and its associated crosstalk NILCO pathways in breast, endometrial, and pancreatic cancers. Leptin and OB-R signaling is linked to increased proliferation, angiogenesis, invasion and migration, and survival of cancer cells. Recent data continues to emerge highlighting the NILCO system components as a key driver of leptin-oncogenic actions in breast, endometrial and pancreatic cancer, specifically in cancer cells that show low or not responsiveness to steroid hormonal cues. High levels of leptin found in obese patients could potentially exacerbate NILCO impact on cancer progression and could also modify the tumor microenvironment. NILCO could be the integration of leptin-induced proliferation, angiogenic and inflammatory actions affecting several cancer types.

In the last 5 years, leptin signaling crosstalk has also become a focus in the area of cancer stem cell development and chemotherapy resistance. However, little data exist detailing these mechanisms in breast, pancreatic and endometrial cancer. The stemness effect of leptin signaling could play an important role in cancer recurrence and drug resistant, and therefore, warrants more intense research. This review highlights how the direct association between obesity, high levels of leptin, and NILCO signaling could induce the progression of cancer. Inhibition of leptin and NILCO signaling could lead to the development of new adjuvant therapies to reduce or eliminate the impact of obesity on cancer.

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REVIEW

Quo vadis motor neuron disease?

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Abstract

Motor neuron disease (MND), also known as amyotrophic lateral sclerosis, is a relentlessly progressive neurodegenerative condition that is invariably fatal, usually within 3 to 5 years of diagnosis. The aetiopathogenesis of MND remains unresolved and no effective treatments exist. The only Food and Drug Administration approved disease modifying therapy is riluzole, a glutamate antagonist, which prolongs survival by up to 3 mo. Current management is largely symptomatic/supportive. There is therefore a desperate and unmet clinical need for discovery of disease mechanisms to guide novel therapeutic strategy. In this review, we start by introducing the organizational anatomy of the motor system, before providing a clinical overview of its dysfunction specifically in MND. We then summarize insights gained from pathological, genetic and animal models and conclude by speculating on optimal strategies to drive the step change in discovery, which is so desperately needed in this arena.

Key words: Motor neuron disease; Amyotrophic lateral sclerosis; Neurodegeneration; Disease models

Core tip: Motor neuron disease (MND) is a fatal neurodegenerative disorder with no known cure. Here we discuss the organization of the motor system and the clinical presentation of MND. We detail the diagnostic criteria for MND including electrophysiological studies and potential future diagnostic markers of disease. We discuss the staging of disease progression in MND. We

then provide an overview of disease management and end with insights into molecular pathogenesis of the disease and the use of disease models.

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ORGANIZATIONAL ANATOMY OF THE MOTOR SYSTEM

The staggering complexity of the vertebrate nervous system is directed largely at the generation and regulation of movement by the careful choreography of muscles responsible for walking, talking and breathing. The motor system can be categorized most simply into upper and lower divisions. Betz cells within both frontal lobe motor cortices are classically large pyramidal upper motor neurons (MNs). Their smaller cortical counterparts densely populate the motor and premotor cortices. Upper MNs control lower MNs in the spinal cord either directly (monosynaptic input) or indirectly (through spinal interneurons). Descending MNs in the spinal cord travel in laterally partitioned corticospinal tracts, most of which cross the midline at the level of the lower brainstem medullary pyramids to synapse contralaterally within the spinal cord. There are also anterior corticospinal tracts, which do not cross at the medullary pyramids but remain ipsilateral. Notably, a minority of spinal cord regions are innervated by these anterior corticospinal projections, which branch and innervate on both sides of the spinal cord, crossing at the appropriate spinal segment. Direct synaptic connection between upper and lower MNs is likely a recent development in evolution, given that it is exclusive to higher primates.

Lower MNs are anatomically positioned in the ventral horns of the spinal cord and motor nuclei within the brainstem; these in turn synapse at neuromuscular junctions and muscle spindles forming a final common pathway for voluntary movement. Spinal MNs are large, polarized cells with long axons, and are the conduit through which the motor cortex in the brain activates contraction of skeletal muscles. These multipolar cells can project axons over a meter long and each innervate up to 1000 muscle fibres. Remarkably, their extensive dentritic arborisation can accommodate up to 10000 synaptic terminals, receiving input from descending upper MNs and spinal interneurons. Despite certain generic properties, distinct molecular phenotypes of MNs exist. Even seemingly simple motor actions require collaboration and coordination of multiple MN subtypes, which are anatomically organized into motor columns and further grouped into motor pools in a muscle-specific manner. The generation of MN subtype diversity is an absolute pre-requisite to survival. In total, the human body has more than 100000 spinal MNs, which innervate 600 peripheral muscle targets organized into bilateral pairs. MNs can be classified according to the type of motor unit they generate into alpha, beta, and gamma. Alpha MNs abound in the motor system and innervate extrafusal skeletal muscle to generate contractile force and movement. Alpha MNs can be further codified by the contractile properties of muscle fibers they innervate into fast-twitch fatigable, fast twitch fatigue resistant, and slow twitch fatigue resistant[1]. Beta MNs innervate both intra- and extrafusal fibres, although these are the least well-understood MN class. Gamma MNs innervate intrafusal muscle fibers of the spindle, modulating their sensitivity to stretch[2,3]. Compared to alpha MNs, gamma MNs possess smaller cell somae, slower axonal conduction velocities, less complex dendritic arrangements and they lack monosynaptic input from proprioceptive sensory neurons[4-8]. This degree of structural and functional diversity commands distinct developmental lineage restriction programs for each different class of MN.

MN subclasses are spatially allocated into groups that reflect both their developmental origins and also their adult function. This coupling of developmental origin to adult function is depicted in Figure 1. MNs are developmentally partitioned into discrete motor columns, which extend along the rostro-caudal (R-C) neural tube. Within a column, the group of MNs responsible for innervating a single skeletal muscle is termed a motor pool, each of which is also arranged by an anatomical logic related to the muscle target(s) of its projections. The medial motor column (MMC) contains MNs that innervate dorsal epaxial muscles, which mainly subserve postural functions. Hypaxial motor column (HMC) MNs project to the ventral hypaxial muscles, which are mainly involved in respiration. The lateral motor columns (LMC) are responsible for innervating limb muscles. The preganglionic motor column (PGC) is present at thoracic levels and MNs originating from here innervate sympathetic ganglia. The MMCs run throughout the R-C extent of the spinal cord, while the LMCs, HMCs and PGCs occur only at brachio-lumbar (LMCs) and thoracic (HMCs and PGCs) foci (Figure 1). Against this background, the simple term "MN" thus fails to capture myriad subtype differences including rostrocaudal position, motor column and axonal trajectory. This striking complexity is an absolute pre-requisite to normal motor function.

MN DISEASE - A CLINICAL PERSPECTIVE

MN disease (MND) causes progressive MN degeneration in the anterior horn of the spinal cord, brain stem and motor cortex[9-12], invariably leading to fatal paralysis usually through respiratory failure[13,14]. The lifetime risk of MND is 1:400 in those of European ancestry[15]. Most cases (90%) are sporadic and affect men more than women. It can present at any age, but with a peak incidence in the sixth to seventh decades of life. Familial

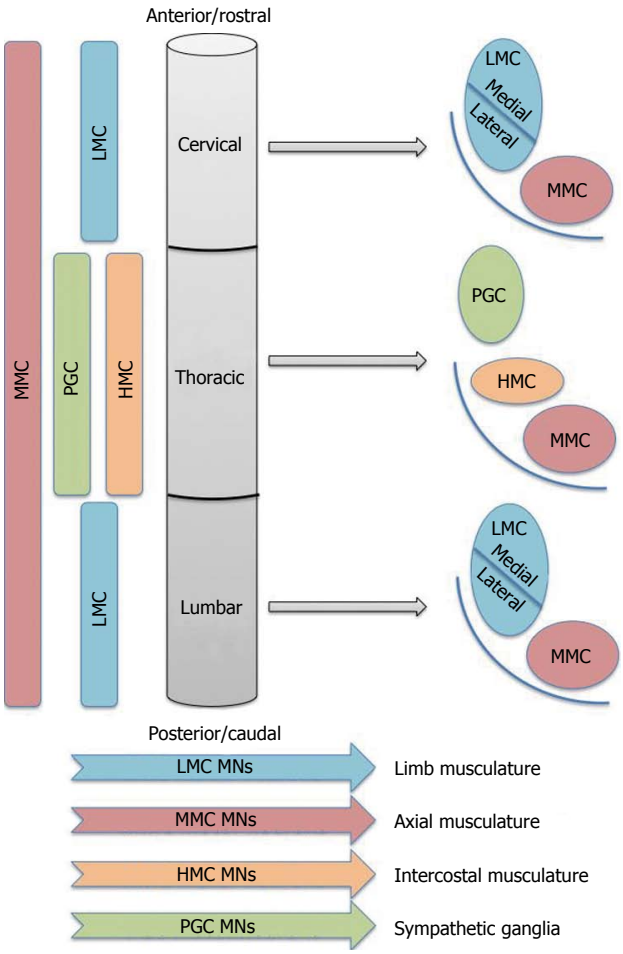

Figure 1 The motor columns of the spinal cord. The LMCs innervate the muscles of the upper and lower limbs, the MMC innervates axial musculature and the HMC and PGC are in the thoracic spinal cord and innervate the intercostal musculature and sympathetic ganglia respectively. LMC: Lateral motor column; MMC: Medial motor column; HMC: Hypaxial motor column; PGC: Preganglionic motor column; MNs: Motor neurons.

MND is caused by mutations in a variety of genes, about 60% of which are now identified[16-18]. Clinically, the patient history and examination typically suggest evidence of upper and lower MN dysfunction in the absence of sensory or autonomic symptoms or signs. A striking clinical feature of this condition is the near universal sparing of the oculomotor nerves and the MNs in the sacral spinal cord that are responsible for pelvic sphincter control, called Onufrowicz nucleus.

Although initial presentation is quite variable, limb muscle weakness often begins focally (over 60% of cases, approximately equally distributed over upper and lower limb) and spreads in an orderly/stereotyped fashion, although overall patterns of motor weakness do vary quite widely between patients. While not pathognomonic, the so called "split-hand phenomenon" is certainly a well-recognized feature of MND, clinically presenting as lateral hand muscle atrophy (*i.e.,* thenar eminence and first dorsal interosseous) with comparative normality of the medial hand muscles. Approximately 30% of patients present with bulbar symptoms, which include dysarthria, dysphagia and sialorrhoea. Sialorrhoea is caused by inability to swallow secretions due to a combination of tongue spasticity, weakness of the facial, mouth and pharyngeal muscles, and loss of oropharyngeal co-ordination and function[19]. Pseudobulbar palsy is also a recognized feature of MND, which can manifest clinically with spasticity of the tongue or of speech, a brisk jaw jerk, a positive gag reflex and mood incongruent emotionality. Muscle cramps and hypersalivation are common symptoms, and head drop, bilateral tongue wasting and widespread fasciculations important physical signs. Fasciculations can be a prominent and early sign in the disease[20]. Although only a minority of patients with MND initially present with acute respiratory failure, the majority do progress to this; indeed it is often the cause of their ultimate demise. The El Escorial criteria can facilitate diagnosis of MND. Combined upper MN and lower MN dysfunction can be difficult to detect in early disease, sometimes explaining diagnostic uncertainty both between conceivable differential diagnoses (Table 1) and different MND subtypes (Table 2). Although a period of observation can be valuable for diagnostic clarification in this context (as concurrent upper MN and lower MN involvement will typically become more evident as the disease progresses), one must take into careful consideration the importance of making a timely diagnosis. Most MND patients who present with predominantly upper MN pathology will develop lower MN signs within 3 or 4 years. The clinical diagnosis of MND is usually fairly self-evident, however it is critical not to miss any possible differential diagnoses listed in Table 2, as suggested by the history, examination and paraclinical tests.

The Revised El Escorial diagnostic criteria and the Awaji electrodiagnostic criteria are well established for the clinical diagnosis of MND and evaluate evidence for progressive degeneration of upper MNs and lower MNs in the absence of other disease processes that could explain the clinical findings[21-23]. There are three diagnostic categories: Clinically definite, probable or possible MND. Importantly, the Awaji criteria established equivalent importance of both clinical and electrophysiological findings when detecting chronic neurogenic changes[24]. A study prior to the introduction of the Awaji criteria found that 29% of MND patients died without a diagnosis of definite MND[25]. The Awaji diagnostic criteria have been shown to increase the sensitivity of MND diagnosis[24,26]. As the diagnosis is made on the basis of upper MN and lower MN involvement in bulbar and spinal regions, the addition of electrophysiology for more sensitive detection of lower MN involvement facilitates the diagnosis. Evidence for neurogenic changes on the electromyography (EMG) should be sought[23]. Chronic neurogenic change may be demonstrated by motor unit potentials (MUPs) of increased amplitude and duration usually with increased number of phases and decreased motor unit recruitment or using a narrow pass filter to detect unstable or complex MUPs. Fibrillation potentials with positive sharp waves may be observed and fasciculation potentials with complex morphology,

Table 1 Possible differential diagnoses and diagnostic clues to discriminate from motor neuron disease[23]

Alternative diagnosis	Diagnostic clue
Cervical (myelo) neuropathy	Cervicalgia, osteopaenia/osteoporosis, abnormal cervical MRI
Benign fasciculations	Absence of weakness, limited distribution, young age
Nutritional (B12 or Cu deficiency)	Usually have sensory impairment
Motor predominant CIDP	Relapsing-remitting course, evidence of demyelination on NCS, IVIG-responsive
Multifocal motor neuropathy with conduction block	Weakness with little wasting, distal and slowly progressive, absent bulbar involvement, conduction block on NCS
Autoimmune and paraneoplastic	*e.g.*, stiff person's syndrome: GAD, amphiphysin, gephyrin antibodies, EMG differences
HIV, HTLV1	HIV: History, sensory neuropathy, opportunistic infections
Parsonage-Turner syndrome (or brachial neuritis)	Preceded by pain, preceding vaccination/viral illness, process arrests and followed by recovery, usually upper limb
Inclusion body myositis	Distribution - forearm and quadriceps, raised CK, muscle biopsy
Hirayama's disease	Upper limb, young males from Asia, unilateral, may arrest after a few years
Radiation-induced motor neuropathies	History and distribution of radiotherapy
Kennedy's disease	Family history (X-linked), gynecomastia
Spinal muscular atrophy	Only affects lower MNs
Primary progressive multiple sclerosis	MRI and/or cerebrospinal fluid (oligoclonal bands)
Adrenoleucodystrophy	Family history (X-linked), adult onset, slowly progressive, usually have sensory ataxia and sphincteric involvement
Hexosaminidase A deficiency	Family history, dystonia, ataxia, psychosis
Poliomyelitis or post-polio syndrome	Clinical history and NCS/EMG
Hereditary spastic paraparesis	Family history and genetic testing

Cu: Copper; CIDP: Chronic inflammatory demyelinating polyneuropathy; NCS: Nerve conduction studies; IVIG: Intravenous immunoglobulin; GAD: Glutamic acid decarboxylase; EMG: Electromyography; HIV: Human immunodeficiency virus; HTLV: Human T-cell lymphotropic virus; MRI: Magnetic resonance imaging; CK: Creatinine kinase.

Table 2 Motor neuron disease subtypes, discriminating features and possible differential diagnoses

MND subtype	Clinical features	Possible differential diagnoses
ALS	Affect both upper MNs and lower MNs Onset 50 or 60 s Median survival 3 to 5 yr	Cervical myeloneuropathy HIV
PLS	Only affect upper MNs 3 yr from onset Onset 50 s Profound spasticity Progressive quadriparesis Late cranial nerve involvement Rarely bulbar onset Slow progression Median survival 5 to 10 yr	Cervical myelopathy Nutritional (B12 or Cu deficiency) Primary progressive multiple sclerosis Hereditary spastic paraparesis Stiff person syndrome Tropical spastic paraparesis (HTLV1) Adrenomyeloneuropathy Hexosaminidase A deficiency Corticobasal degeneration
PMA	Only affect upper MNs 3 yr from onset Focal asymmetric distal weakness, followed by proximal involvement Late bullar/respiratory involvement Earlier onset than ALS Raised CK (< 10 × normal) Median survival 3 to 5 yr	Benign fasciculations Post-polio syndrome Adult onset spinal muscular atrophy Inclusion body myositis

HIV: Human immunodeficiency virus; Cu: Copper; CK: Creatinine kinase; ALS: Amyotrophic lateral sclerosis; PLS: Primary lateral sclerosis; HTLV: Human T-cell lymphotropic virus; PMA: Progressive muscular atrophy.

in the presence of chronic neurogenic change on needle EMG, may also be seen. The Revised El Escorial and Awaji criteria have proved very useful for diagnosis, especially for determining patient inclusion for clinical trials, however for use in clinical practice it is proposed that these criteria should be updated, to reflect the phenotypic heterogeneity of MND, the stage of disease and the presence of familial disease[27].

Similarly the use of investigations to support upper MN involvement would add further diagnostic certainty. Transcranial magnetic stimulation (TMS) is a technique used to measure corticomotoneuronal function with the parameters of motor threshold, motor evoked potential amplitude, central motor conduction time, cortical silent period, intracortical inhibition and facilitation[28]. Early cortical hyperexcitability, which may reflect glutamate excitotoxicity, precedes lower MN involvement in MND, and through the course of the disease this hyperexcitability decreases[28-32]. Threshold tracking TMS has the potential for use as a diagnostic marker and distinguishes MND from non-MND disorders with a sensitivity of 73.21% and specificity of 80.88% at an

early disease stage[33]. Three hypotheses for MN death have been proposed: (1) a "dying-forward" phenomenon, where diseases initiates in upper MNs, leading to excitotoxic death of lower MNs; (2) a "dying-back" phenomenon, where disease begins at the lower MN level and progresses back to the upper MNs; or (3) an independent-degeneration phenomenon. The finding that cortical hyperexcitability starts below lower MN involvement supports the "dying-forward" hypothesis. Furthermore neuroimaging techniques, such as diffusion tensor magnetic resonance imaging, are showing promise for determining motor cortex and corticospinal tract involvement in disease, and could be used as biomarkers of disease and predictors of prognosis[34].

Various staging systems have been devised to measure disease progression in MND[35-40]. Individuals can progress through the disease at very variable rates[41,42], and as each clinical stage is reached at a consistent proportion through the disease process, staging can be used to make more useful comparisons between patients[35,36]. Furthermore, incremental stages correspond to decreasing function and health utility, and can be used in cost-benefit analyses of new treatments[38]. An important application of staging is as an endpoint in clinical trial design. The goal is to develop therapies which would prolong time in the earlier stages of disease, when function and quality of life are better, as compared to the later stages.

Cognitive impairment is recognized in up to half of patients with MND, usually detectable on neuropsychological testing rather than from routine clinical evaluation. However, frank dementia of the frontotemporal lobar degeneration (FTLD) type is increasingly diagnosed against the background of pathological and genetic discoveries that have mechanistically linked these two conditions together over the last decade[43]. Conversely, some patients presenting with FTLD will have clinical and para-clinical evidence of MND and the mode of presentation here is likely determined by the same pathomechanistic process starting/predominating at different neuraxial sites. Approximately 15% of MND patients have a clinical diagnosis of FTLD and 15% of FTLD patients have a diagnosis of MND[43,44].

Both European Federation of Neurological Societies and American Academy of Neurology guidelines for the management of MND patients have guided management to some degree in the United Kingdom[45-48]. Following a review decision in November 2014, the national institute for health and care excellence (NICE) is currently developing a guideline for the management of MND. This will ultimately replace the current NICE guideline on non-invasive ventilation in MND. The MND Association website offers a comprehensive list of available regional and national/international guidelines in specific MND-related areas, with direct links to documents. Indeed the support of the MND Association in all respects is frequently fed back as being highly valued by patients and carers. Most patients will experience hypoventilation/orthopnea as the disease progresses, justifying proactive interval monitoring of respiratory performance (including nocturnal oximetry, dynamic forced vital capacity, and maximal inspiratory pressure). Noninvasive positive pressure ventilation should be accessible when needed. Importantly, the management of MND should be in a multidisciplinary clinical setting, including experts in neurology, respiratory medicine, nutrition, psychology/psychiatry, speech therapy, physical and occupational therapy, social work, and case management. Other supportive measures include reactive and proactive interval examination of swallowing function as MND increases risk of aspiration. It is noteworthy that parotid/submandibular botulinum toxin injections can be helpful for sialorrhoea[19,49]. Consideration of a percutaneous gastrostomy tube can help to maintain body weight and hydration in MND. Pseudobulbar affect is often treated off-licence with selective serotonin reuptake inhibitors or tricyclic antidepressants. In October 2010, Food and Drug Administration approved a dextromethorphan-quinidine combination for symptomatic relief of pseudobulbar affect.

LESSONS FROM PATHOLOGICAL, GENETIC, ANIMAL AND CELLULAR MODELS

Various experimental strategies including *in-vivo* studies, cell based *in-vitro* approaches and human post-mortem neuropathological specimens from MND patients have been employed in order to improve understanding of this disease. Human stem cell strategies are becoming an increasingly important component of the armoury of investigative tools used to study disease mechanisms and identify potential therapeutic targets[50,51].

Historically, the most intensively studied cause of familial MND has been mutations in the copper/zinc superoxide dismutase (*SOD1*) gene, which account for approximately 15% of cases of familial MND and less than 5% of sporadic MND cases. The mutant SOD1 protein characteristically maintains its dismutase function, but appears to cause MN degeneration through alternative mechanisms, including a possible toxic gain of function[52]. Well over 100 individual point mutations located throughout the primary structure of SOD1 are sufficient to cause disease, suggesting protein-folding abnormalities as a possible initiating event. Transgenic mice globally expressing mutant forms of human SOD1 exhibit selective MN degeneration, which broadly mirrors the pathology of human sporadic and familial MND. Unfortunately, despite countless pre-clinical and clinical trials based on SOD1 models, not one of these has led to a significant therapeutic advance in MND. A landmark study in 2006 then discovered that the pathological hallmark of > 95% MND cases (sporadic and familial) is cytoplasmic misaccumulation of ubiquitinated and hyperphosphorylated transactive response DNA-binding protein (TDP-43)[53], a highly conserved, ubiquitously expressed and multifunctional nuclear protein with

both DNA and RNA binding capacities[54-56]. A striking observation made in this work was that TDP-43 appeared mislocalised from the nucleus to the cytoplasm in MND and FTLD, although the pathophysiological significance of this remains incompletely understood. Interestingly, TDP-43 immunoreactive inclusions are found in both neurons and glia in MND and FTLD, hence their proposed taxonomic reclassification as TDP-43 "proteinopathies". SOD1 mutations do not produce this common hallmark of MND and may not therefore be pathomechanistically representative of the majority of MND. Different subtypes of FTLD are based upon the protein found in pathological inclusions: In 45% of cases this is TDP-43, in another 45% of cases this is tau, and in 10% of cases this is fused in sarcoma (FUS)[43,57].

Other recent discoveries identified MND-causing gene mutations in TDP-43 and FUS[58,59]; findings that both complement and extend previous pathological studies. Furthermore, two recent contemporaneous studies have identified another MND-causing intronic mutation that introduces long hexanucleotide repeats into *C9orf72* pre-mRNA[60,61], which is the most frequent genetic cause of MND and a common cause of FTLD. TDP-43 and FUS are both RNA-binding proteins. Collectively, these discoveries implicate a dysregulation of RNA metabolism as playing a crucial role in MND pathogenesis. In addition to these genes, several further mutations have been discovered including in the following genes: *PGRN, UBQLN2, SQSTM1, PFN1, ANG, VCP, MATR3, TUB4A*. Taken together, gene mutations and pathological studies implicate both protein misfolding/aggregation and perturbed RNA regulation as key underlying pathways in the molecular pathogenesis of MND[43,58,59,62-66].

A widely held view regarding the pathogenesis of neurodegenerative disease posits that selective injury to a disease-specific subclass of neurons is mechanistically cell autonomous. This "neuron-centric" view has been increasingly challenged by pivotal mice-chimera studies using lineage-specific expression of mutant SOD1 and subsequent related investigation, which confirmed a major non cell-autonomous role for astrocytes and microglia in SOD1-related MND pathogenesis[67-69]. Non cell-autonomous injury has also recently been implicated in sporadic MND, raising the possibility of common pathogenic mechanisms[70,71].

The discovery of induced pluripotent stem cells (iPSC) enables patient-specific fibroblasts to be virally transduced with up to 4 transcription factors and "reprogrammed" into embryonic-like stem cells[72]. Using insights from developmental neurobiology, these cells can subsequently be treated with a programme of extrinsic cues to direct their differentiation into a range of regionally defined neurons and glia for further study[73-76]. Importantly, a variety of studies have confirmed the capacity of these terminally differentiated cells to recapitulate key pathological hallmarks of a range of different neurodegenerative diseases[71,77-79]. In particular, several important studies have already demonstrated that iPSC-derived neurons and glia from patients with monogenic and sporadic MND show pathological phenotypes when compared to their control counterparts. Furthermore, this reductionist and human *in vitro* model system allows assays that directly elucidate non cell autonomous mechanisms of disease[80]. Several studies have also confirmed the utility in this model system as a pre-clinical test-bed for drug discovery[81-83], including the practical feasibility of high throughput automated approaches[84].

FUTURE STRATEGIES

We conclude that the integration of human experimental approaches is required to drive the desperately needed discovery of disease mechanisms and therapeutic strategy in MND. Unfortunately animal models have failed to deliver a significant therapeutic advance in MND, despite numerous efforts and important discoveries. Human iPSC models can better approximate clinical MND not only by virtue of species, but also because they express mutations at accurate pathophysiological levels and thus bypass the need for artificial overexpression, knock down or knock out experiments. A multitude of studies have now validated the human iPSC technology for disease modeling of both developmental and adult-onset conditions and drug discovery. However, this remains an *in vitro* system and thus lacks the dynamic cellular and signaling environments of an *in vivo* model. The integration of transgenic animal models that recapitulate MND pathogenesis together with patient-specific iPSCs represents an unprecedented opportunity to capture the complexity of pathogenic events underlying this devastating condition. By combining these approaches at the pre-clinical phase, we firmly believe that the translational yield of clinical trials will increase in MND.

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REVIEW

Vascular targeted photochemotherapy using padoporfin and padeliporfin as a method of the focal treatment of localised prostate cancer - clinician's insight

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Abstract

Vascular targeted photochemotherapy (VTP) holds promise as a novel strategy of the focal treatment of localised prostate cancer (LPCa). It is convenient to perform, minimally invasive and can be conduct in ambulatory conditions. In this review, methodologic aspects of padoporfin- and padeliporfin-mediated VTP and its clinical application in focal treatment of LPCa as well as future perspective of this method were presented. Physicochemical and pharmacokinetic parameters of padoporphin and padeliporfin using as VTP photosensitizers were described, as well as methodologic question of radiation delivery and dosimetry, and oxygen monitoring in cancer tissue in context of VTP safety and efficiency of LPCa focal therapy were discussed. The results of clinical trials concerning application of padoporfin- and padeliporfin-mediated VTP in LPCa were also presented. The future of VTP is development of protocols, founded on the real-time feedback and rules-based approach to make this strategy a standard procedure in LPCa treatment. To evaluate clinical potential of this procedure, a cost-effectiveness analysis is also necessary.

Key words: Localised prostate cancer; Focal therapy; Vascular-targeted photochemotherapy; Methodology; Clinical trials

Core tip: Vascular targeted photochemotherapy (VTP) represents new paradigm in focal therapy of prostate cancer (PCa). Physicochemical, pharmacodynamic and pharmacokinetic properties of padoporfin and padeliporfin, which are palladium derivatives of bacteriochlorin, make them suitable photosensitizers for VTP. Good visualisation of tumours and selective targeting of tumour lesion are mandatory for VTP to be efficient. Results of clinical trials confirm safety and efficiency of VTP in treatment of PCa. New protocols are necessary to make VTP standard method of PCa therapy.

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INTRODUCTION

Prostate cancer (PCa) is the fourth most prevalent cancer in the World and the second most frequently diagnosed cancer in men. In 2012, this cancer was the fifth dominant cause of cancer death in men, even though a measurement of prostate specific antigen (PSA) levels following biopsy allows to discover prostate cancers at early stages, suitable to therapy[1,2].

In conventional therapy of PCa, the target of treatment is an entire organ seized by malignant lesions, contrary to many other tumours, such as ovarian, cervical or colorectal cancer[3]. The currently available treatment modalities for localised PCa (LPCa) are radical therapy (RT) and active surveillance. RT consists in ablation of entire prostate gland[3-6] while active surveillance includes monitoring of serum PSA concentration and repeat prostate biopsies to select patients for curative therapy[6-8]. Strategies of RT, including prostatectomy, external beam radiotherapy and brachytherapy, can result in substantial genitourinary and rectal adverse effects, that, as a consequence, damage surrounding tissues and deteriorate patients quality of life[4,9-11], whereas active surveillance presents a considerable risk of cancer progression, metastases, and patients mortality[6,12-14]. There is no difference of long-term PCa mortality (< 3%) after robotic prostatectomy comparing to that observed during 12 and more years of active surveillance[15]. Moreover, frequent medical exams during active surveillance may create adverse effects and deteriorate patient quality of life[6]. This situation gives an impetus to search new options of PCa treatment. In the last few years, the interest in focal therapy of localised PCa has increased[16].

Focal therapy is based on the conception of LPCa treatment by destruction of only cancerous lesions localised in prostate gland to spare remainder of this gland and, in this manner, to minimise morbidity[17-20]. In the case of multifocal tumours, only the index lesion, which is generally defined as the largest-volume lesion with the highest grade, is predictive of progression[3,21-24], although there are no maximum tumour volume over which focal therapy is recommended[19,25]. The strategies of prostate focal ablation may target directly the lesions identified as malignant (targeted ablation), a part of the gland that is known to harbour malignancy (zonal ablation), or a lobe along with ipsilateral neurovascular bundle (NVB) with preservation of contralateral and its NVB, involving ablation of urethra as natural boundary of ablation (hemiablation)[19,20].

The primary aim of focal therapy of LPCa is to reduce trauma to the cavernous nerves, resulting in less erectile dysfunction. The method which may adequately spare a sufficient component of the NVB, so that potency is preserved, is vascular targeted photochemotherapy (VTP), usually regarded as a form of the focal photodynamic therapy (PDT)[24,26,27].

PDT IN FOCAL TREATMENT OF PCa

In general, PDT is a minimally invasive treatment procedure, involving optical radiation (usually called "light"), oxygen, and radiation-sensitising dye, termed photosensitizer. The PDT action is based on light activation of photosensitizer localised in target tissue producing reactive oxygen species which destroy target cells though direct cytotoxicity, vascular shutdown and activation of an immune response[28-30].

The development of prostate PDT in particular has accelerated rapidly in past few decades[31-33]. Some clinical studies of prostate cancer PDT with use of transurethral or transperineal irradiation and many of photosensitizers or their precursors, such as haematoporphyrin derivative[34], meso-tetra-(m-hydroxyphenyl)chlorin[35,36], 5-aminolevulinic acid[37], motexafin lutetium (Lu-Tex)[38-40] or temoporfin[41], were conducted. The protocols of these studies were grounded on the conception of cellular-targeted photochemotherapy (CTP), in which photosensitizer after administration is preferentially accumulated in parenchymal cells of tumour and causes their damage through production of singlet oxygen when activated by radiation[42,43]. This strategy is characterised by a long interval between administration of photosensitizer and its activation with radiation [drug-light interval (DLI)] that makes this treatment uncomfortable for patients[27,44]. Moreover, CTP of prostate cancer may cause adverse effects related to both photosensitizer pharmacokinetics, such as prolonged photosensitivity, and treatment protocol, such as haematuria, infections, incontinence or prostate oedema[35,45]. Finally, this strategy does not preserve adjacent structures of prostate gland, such as NVB, rectum or urinary sphincter[27,45,46].

In this situation, a VTP as an alternative method of LPCa treatment has been proposed. In this treatment modality, the photosensitising agents following intravenous administration, remain only in the circulation until elimination from organism, with minimal or no extravasation[47-50]. Under these conditions, the VTP mediated oxidative stress is strictly limited to vascular compartment, leading to tumour cell death due to vascular occlusion and shutting down the tumour blood supply[47,51,52]. In VTP oxidative stress is mediated through superoxide radical anion, hydrogen peroxide, hydroxyl radicals and secondary reactive nitrogen species, such as nitrogen oxide, as opposed to conventional PDT, involving singlet oxygen production[53]. Therefore, some authors claimed that VTP represents a new paradigm in focal therapy of LPCa. The two novel

bacteriochlorins, padoporphin and padeliporphin, are used as photosensitizers in this treatment strategy[54,55].

PADOPORPHIN AND PADELIPORPHIN - PHOTOSENSITIZERS USED IN FOCAL VTP OF LPCa

Padoporfin (palladium bacteriopheophorbide; WST-09; Tookad®) is not soluble in water; its logP (Briggs' logarithm from octanol-water partition coefficient P, commonly used as a measure of molecular lipophilicity), is 1.38[55]. Preparing of water-soluble formulations of padoporfin for intravenous administration requires the use of solubilising agents such as Cremophor® in which photosensitizer undergoes aggregation upon injection but rapidly disaggregates in blood plasma[56,57]. Pharmacokinetic study using murine models showed that padoporfin in Cremophor® formulation, after bolus administration to healthy mice at a dose of 6 mg/kg[47] or to EMT-6 breast cancer-xenografted mice at a dose of 5 mg/kg[58], is eliminated in the two-step process from circulation of animals. The alpha- and beta-half lives are longer in the case of tumour-bearing mice (2 min and 1.3 h, respectively), comparing to these in the healthy animals (0.6 and 11 min, respectively)[47,58,59]. The total rate of clearance and the apparent volume distribution of padoporfin in the tumour bearing mice are estimated to be 11 mL/h and 0.9 mL respectively[58] while the maximal plasma concentration (C_{max}) is 19 mg/L, at the maximal plasma concentration time (t_{max}) of 5 min[58]. Similarly, in canine models, the maximum concentration of padoporfin in circulation after intravenous injection occurred in less than 10 min[60]. Padoporfin binds in 30% with human low level density lipoproteins (LDL) and in 50% with human high density lipoproteins (HDL) while its binding with human high density proteins (HDP), containing human serum albumins, is lower (about 15%)[61].

In contrast, padeliporfin (palladium bacteriopheophorbide monolysine taurine; WST-11; Tookad®Soluble; Stakel®) is soluble in aqueous solutions; its logP is -0.19[55,62]. After intravenous bolus injection at a dose of 6 mg/kg into healthy mice, this photosensitizer is eliminated from their circulation in one-step process, with half-life 1.65 min, apparent volume of distribution 2.12 mL and with rate of clearance 0.89 mL/min[55]. The C_{max} of padeliporfin is about 52 mg/L, at a t_{max} of 2 min. Within 5 min after injection, about 90% of administered dye is eliminated from mouse circulation, and 30 min after injection, the photosensitizer concentration in blood plasma reaches practically the background levels[47]. In order, after 20 min intravenous infusion at a dose of 10 mg/kg into healthy rats, the half-life of padeliporfin was 7.5 min[47]. Padeliporfin is characterised by minimal extravasation from vasculature and therefore remains in circulation even at high doses[47,63]. Contrary to padoporfin, padeliporfin binds primarily to HDP (about 80%) and poorly to LDL and HDL (5% and 15%, respectively)[61]. Preclinical studies in animal models showed that padeliporfin-mediated photosensitisation causes occlusion of the full tumour vasculature in a few minutes of treatment[50,54,55,64]. Thus, padeliporfin appears to have a higher therapeutic index and to be far easier to use in the VTP treatment of prostate cancer, comparing to padoporfin[27].

As padoporfin and padeliporfin in hydrophilic media present absorption maxima in the IR-A region (700-2500 nm)[55,65], therefore, for activation of these photosensitizing agents in VTP, the radiation at a wavelength of 763 nm and 753 nm, near to absorption maxima of these photosensitizer, is usually used[66,67]. The advantage of this radiation is its considerably deeper penetration into human prostate tissues (0.57 cm for λ = 763 nm) comparing to red light (about 10 mm for λ = 633 nm), usually used in conventional PDT[68,69].

In the clinical conditions, photosensitizers are usually delivered in form of infusion injected using a syringe pump through intravenous delivery line[70]. For delivery of padoporfin, which is not soluble in water, Weersink *et al*[70] used an aqueous formulation containing benzylic alcohol, ethanol, and Cremophor®, adjusted to pH 7.4. Due to the possibility of interactions between polyvinyl chloride (PVC) and Cremophor®, the syringe for injection and lines for intravenous photosensitizer delivery were PVC-free[70].

The short t_{max} of padoporfin and padeliporphin implies short DLI value (10 min for both theses dyes), as was shown in clinical studies[44,56,57,66,71,72]. This suggests that administration of drug and radiation may be conducted in one clinical visit[44]. In addition, due to the rapid clearance of these agents from circulation, which decreases a risk of delayed skin photosensitisation, the patients can often be discharged on the day of treatment, without long-term sunlight protection[44,66].

Monitoring of padoporphin and padeliporphin in serum is complicated, because, contrary to many other photosensitizers, they present extremely weak fluorescence, and therefore they cannot be monitored using standard fluorescence techniques[47]. For serum monitoring of these photosensitizing dyes, inductively coupled plasma mass spectrometry[47], graphite furnace atomic absorption at a wavelength 247.6 nm (palladium atomic absorption line)[58] or *in situ* absorption spectroscopy after serum protein ultracentrifugation[61] may be used, however, in clinical practice, the effect and not concentration of these drugs are usually monitored during VTP[62].

Selected physicochemical and pharmacokinetic properties of padoporfin and padeliporfin are presented in Table 1.

RADIATION DELIVERY AND MONITORING

For effective irradiation of the prostate gland, the fluence of radiation must be suitable to treat the whole

Table 1 Selected physicochemical and pharmacokinetic properties of padoporfin and padeliporfin

Parameter	Value	
	Padoporfin	Padeliporfin
logP (octanol-water)	1.38[55]	-0.19[55]
Apparent volume of distribution (mL)	[1]0.9[55]	[2]2.12[55]
Alfa half-life (min)	[1]1.86[58]	[2]1.65[55]
Beta half-life (h)	[1]1.3[58]	-
Total body clearance (mL/min)	[1]0.18[58]	[2]0.89[55]
Maximal plasma concentration (mg/L)	[1]19[58]	[2]52[47]
Maximal plasma concentration time (min)	[1]5[58]	[2]2[47]
Plasma LDL binding (%)	[3]30[61]	[3]5[61]
Plasma HDL binding (%)	[3]50[61]	[3]15[61]
Plasma HDP binding (%)	[3]15[61]	[3]80[61]
Standard intravenous drug dose (mg/kg)	2[66]	4[61]
Standard radiation fluence (J/cm)	200[83]	200[66]
Drug-light interval (min)	10[66]	10[66]
Standard irradiation wavelength (nm)	763[66]	753[66]

[1]Results obtained for mice with transplanted breast tumor cells after 5 mg/kg intravenous injection; [2]Results obtained for healthy mice after 6 mg/kg intravenous injection; [3]Results obtained for human protein fractions *in vitro*. LDL: Low level density lipoprotein; HDL: High density lipoprotein; HDP: High density protein.

volume of target tissue, and, at the same time, to spare surrounding organs of the prostate, whose functioning is essential for life[70]. Furthermore, penetration and distribution of radiation into prostate gland strongly depends on tissue optical absorption and scattering. In these conditions, interstitial light delivery in prostate cancer VTP has been proposed in which radiation is delivered *via* transperineal optical fibres positioned under transrectal ultrasound (TRUS) or magnetic resonance imaging (MRI) guidance, analogously to the procedure used in brachytherapy[70,73].

The number of fibres as well as characteristics of light sources and their positions depends on volume and shape of each prostate and on area of lesion. In the case of human prostate, up to six optical fibres may be necessary to ensure a full distribution of IR-A radiation throughout the prostate gland[70]. In this situation, radiation fluence is usually calculated as energy into unit of fibre length (J/cm) and not into unit of exposed surface (J/cm^2). The ratio of the total length of used fibres in cm to the planned treatment volume (PTV) of targeted prostate tissue in milliliter, is termed light density index (LDI) and plays an important role in optimisation of radiation delivery during VTP of LPCa[67,74].

The radiation fluence and position of fibres can be either planned in advance, on the basis of imaging data and knowledge of optical properties of tissues within prostate, or adjusted during the treatment, with use of dose rate standards, placed at the prostate boundary. In clinical practice, a combination of both these approaches is often used, with treatment plans to determine the prostate size and shape and with intraoperative adjustment, to estimate optimal fibre number, length, position and radiation dose. According to currently prescribed VTP regimens, interstitial irradiation should be conducted in a darkened room to prevent cutaneous photosensitisation, however, rapid elimination of padoporfin and padeliporfin from organism decreases the necessity to avoid a long-period sunlight exposure[75].

Intraperineal irradiation allows to modify radiation fluence during treatment to create more accurate focal treatments and reduce the risk of adverse effects by minimising light delivery to the urethra, rectum, and urinary sphincter. Intraoperative contrast-enhanced ultrasound or MRI method may play an important role in monitoring effects of VTP toward treated area[5,70]. Johansson *et al*[76] developed a real-time software tool for optimising time of prostate gland irradiation during interstitial VTP. This optimising is based on the continuous monitoring of radiation attenuation in the prostate tissues[76]. However, this method, applied in the steady state without measurement of absolute radiation fluence, cannot indicate variation of photosensitizer and oxygen concentration in the prostate tissue and, at the same time, may not reflect real conditions of VTP course[76]. As shown by Xu *et al*[77], both absorption and scattering coefficients can be determined within 10% for a wide range of optical properties using a quick and precise forward model. This creates the possibility that both radiation fluence and photosensitizer concentration can be determined from the combined steady state and frequency domain measurement and adjust the radiation dose adequately[78]. Irradiation can be applied simultaneously with photosensitizer administration, after ending of this administration or at the point of maximal serum concentration of photosensitizer[60]. For delivery of conformal radiation, the use of functional optical fibres may be necessary. Rendon *et al*[79,80] received some radiation isodose profile using diffusers with tailored longitudinal emission profiles.

OXYGEN MONITORING

Contrary to typical CTP, in the case of VTP the oxygen pressure (pO_2) of the blood is more important for effectiveness of this method than that in the parenchymal cells of tumour. This is advantage of VTP in treatment of prostate cancer, because areas of low pO_2 are documented in the parenchymal cells of malignant prostate gland[46,81]. Monitoring the haemodynamics *in situ* provides the relevant information on oxygen concentration in the blood. In some studies, a blood oxygen level dependent contrast MRI was used to demonstrate a correlation between decrease in blood saturation during irradiation and prostate cancer remission for padoporfin-mediated VTP[51,70]. This imaging method represents spatial and temporal changes in oxygenation, flow and volume of blood[82].

CLINICAL TRIALS

Phase I trials

Weersink *et al*[83] performed study of cutaneous photosensitivity as potential adverse effect of LCaP treatment with padoporfin VTP, as a part of phase I clinical trial

concerning application of padoporfin-mediated VTP in the therapy of prostate cancer. Padoporfin at a dose of 0.1-2 mg/kg was administered to 10 patients with LPCa and, subsequently, the prostate glands were irradiated using diode laser with radiation of 763 nm, through optical fibres placed percutaneously to deliver radiation fluence of 10-360 J/cm in each lobe of the prostate. DLI was 6-10 min, and irradiation time was 17-30 min. For testing skin photosensitivity, at 7-28 d before treatment, the minimum erythema dose in each patient was determined by exposing four square spots on the back to solar radiation simulating lamp without and with ultraviolet (UV) filter, at a fluence from 1 to 128 J/cm^2. The irradiation with full spectrum of solar-simulated radiation source did not increase skin erythema after intravenous administration of padoporfin while after removing UV region from this spectrum with optical filters, no phototoxic effects were observed when skin was exposed to light at a fluence of 128 J/cm^2, and time interval of 1-3 h after photosensitizer administration. These results suggests that cutaneous photosensitivity during padoporfin-mediated VTP of LPCa is negligible under clinical protocol[83].

Phase Ⅰ clinical trial of padoporfin-mediated VTP in 24 patients with locally recurrent PCa after external beam radiotherapy (EBRT), was conducted by Trachtenberg *et al*[56]. In this study, safety of VTP, as well as tumour response to escalating photosensitizer doses and radiation fluences were examined. Both padoporfin doses and radiation fluences at a wavelength of 763 nm were increased to the maximal values of 2 mg/kg and 360 J/cm^2, respectively. The treatment response was evaluated a week after treatment using gadolinium enhanced MRI.

The results of this study revealed strong dependence of prostate cancer response to VTP treatment on photosensitizer dose and radiation fluence. The considerable variability among patients in the response was also observed, even when drug doses and radiation fluences were the same. Only in patients receiving the highest drug/radiation dose, the sizeable necrotic zones (up to 2.2 cm diameter) in tumour tissue were observed. Avascularity regions detectible using MRI 7 d after treatment corresponded to regions of histopathological fibrosis in which no residual viable tumours were apparent. These results suggested the utility of MRI after VTP treatment as an early marker of response[56].

Phase Ⅰ/Ⅱ trials

Gertner *et al*[84] conducted the phase Ⅰ/Ⅱ two-centre clinical trial for assessment of safety, efficiency and pharmacokinetics of padoporfin-mediated VTP in patients with locally recurrent PCa after EBRT. The sensitizer was administered intravenously at a dose of 0.1-2.0 mg/kg and radiation was delivered at a fluence of 100 J/cm of laser delivery fibres. Padoporfin concentration was measured in blood and urine samples and skin photosensitivity was also evaluated. Treatment response was evaluated using measurement of serum PSA level, biopsy and gadolinium-enhanced MRI. The molecules of contrast agent, which contain gadolinium ions, are selectively captured by tumour cells to make these cells more visible in magnetic resonance image and, at the same time, to increase MRI precision and resolution in PCa diagnostics[85]. No photosensitizer adverse effects including skin photosensitivity were observed. There was linear relationship between dose and plasma concentration of padoporfin and no detectable plasma level of this photosensitizer by 2 h after irradiation was detected. No padoporfin concentration in urine at any time point during one week after treatment was observed. The lesion formation in tumour tissues on gadolinium-enhanced MRI was seen. Average depth of the effective penetration of radiation was 5.5 mm in the lesions and 3.2 mm in the selected tissue regions that revealed no MRI response. These results showed that padoporfin mediated VTP is a safe and efficient method of radiation recurrent LPCa treatment. Decrease of effective penetration of radiation in the unresponsive areas may be probably caused by fibrosis and calcification in the tissue of prostate cancer recurrent after radiation[84].

Arumainayagam *et al*[86] undertook the phase Ⅰ/Ⅱ study of drug dose and radiation fluence escalation in the padoporfin-mediated VTP, in patients previously subjected to active surveillance. For this trial, 34 men with gleason score $\leqslant$ 7 and PSA < 20 μg/L were enrolled. The patients received 2 mg/kg padoporfin in a 20 min infusion, following illumination with 763 nm radiation with use of a diode laser. The radiation fluence was increased from 100 to 300 J/cm. Under general anaesthetic, optical fibres within plastic needles were inserted into the prostate upon guidance with use of TRUS and perineal template. The procedure took 120-150 min depending on number of inserted fibres and size of prostate. The VTP-induced necrosis, as a measure of treatment efficiency, was evaluated with use of gadolinium-enhanced MRI, at a week after irradiation. The optimal radiation fluence for producing controllable ablation was 200 J/cm. In patients receiving this fluence of radiation, up to 73% necrosis of the prostate was observed, with sparing of capsule and extraprostatic tissues. The adverse effects on urinary tract were mild and transient. The irritative symptoms, persisting in many patients less than two weeks, were only recognised, whereas no incontinence episodes were reported. In one patient, hypotension caused an adverse cardiac event and a stroke. The authors supposed that observed cardiovascular adverse effects were caused by the formulation of the photosensitizer that is water insoluble and requires Cremophor® for intravenous administration. For this reason, the clinical trial has been withdrawn[86].

A prospective, multicentre, phase Ⅰ/Ⅱ study of the tolerability and safety of one-sided padeliporfin-mediated VTP in patients with LPCa was also completed. Treatment consisted of a single, 10 min, intravenous administration of padeliporfin at doses of 2, 4 or 6 mg/kg, followed by irradiation with laser at a wavelength of 753 nm and fluences of 200 or 300 J/cm. The radia-

tion was delivered for 20 min through transperineal interstitial optical fibres inserted into implant catheters and positioned with use of brachytherapy-like template under guidance of TRUS. Six positive results of biopsies in the month was indication for patient retreatment with padeliporfin-mediated VTP[87].

Azzouzi *et al*[88] conducted a pooled analysis of results obtained for 117 men from one phase Ⅰ/Ⅱ (NCT00946881) and 2 phase Ⅱ (NCT00707356, and NCT00975429) clinical trials with LPCa, PSA < 10 μg/L, and Gleason score ⩽ 7, who received padeliporfin at a dose of 4 mg/kg in 10 min intravenous infusion, following illumination with 753 nm radiation at a fluence of 200 J/cm delivered by transperineal fibres inserted in the prostate under TRUS guidance. Primary outcome was negative biopsies results in the treated lobes during six month after treatment. PSA concentration was determined at 1st, 3th, and 6th month after VTP. Magnetic resonance imaging was conducted at a week, as well as at 3, and 6 mo after irradiation. Furthermore, International Prostate Symptom Score (IPSS), International Index of Erectile Function (IIEF-5) and adverse effects were assessed at 7 d, and at 1, 3, and 6 mo after procedure. In a 6th month, the negative biopsy outcomes were observed in 68.4% of overall examined population (n = 114) and 80.6% of patients treated with hemiablation with LDI ⩾ 1 (n = 67). PSA concentration in both groups decreased by 2.0 μg/L at 6th month of trial and percent of prostate necrosis at a first week of study was 76.5% and 86.3%, respectively. Minor variations of IPSS and IIEF-5 parameters suggested an inconsiderable amelioration of urinary function and an unimportant exacerbation of sexual function. In spite of this fact, patients tolerated this procedure well, and the authors found it to be a promising method of PCa treatment[62].

Phase Ⅱ trials

Trachtenberg *et al*[57] executed phase Ⅱ case study to evaluate efficiency of padoporfin-mediated VTP as a method of ablation of the entire prostate gland in patients with recurrent LPCa after the EBRT failure. Twenty-eight patients enrolled in this trial received a padoporfin dose of 2 mg/kg and a specific radiation fluence (λ = 763 nm), established in computer-assisted treatment plan. A complete response required radiation fluences of at least 23 J/cm^2 in 90% of the prostate volume. An increased radiation fluence ameliorated the tissue response, encompassing up to 80% of the prostate in some patients. Among the 13 patients who received at least this radiation fluence, 8 had negative results of biopsy at 6 mo. Adverse effects were moderate and self-limited in most patients; two patients had recto-urethral fistulae, one of which closed spontaneously. Padoporfin-mediated VTP produced large avascular regions in the irradiated prostate, and caused complete negative-biopsy response at high radiation doses. It enables the treatment of entire prostate gland with minimal damage of surrounding tissues. The results of this study reveal clinical potential of padoporfin-mediated VTP with to treat recurrence of prostate cancer after EBRT[57].

Arumainayagam *et al*[72] achieved hemiablation in 40 patients using padeliporfin-mediated VTP with transperineally delivered radiation. Development of necrosis was assessed using MRI. Only two patients reported urinary retention as adverse effects, however patient quality of life during the treatment was not evaluated[72]. In analogous study of Azzouzi, necrosis was seen with use of MRI in 87% of the treated lobes. Concerning adverse effects, two cases of prostatitis, and single cases of haematuria, orchitis, optic neuropathy, and urethral stenosis were reported[88].

Quoraishi *et al*[74] made multicentre phase Ⅱ clinical trial to optimise conditions of padeliporfin-mediated VTP in LPCa treatment in the case of 40 patients with PSA concentration < 10 μg/L. Photosensitizer was administered intravenously at a dose of 2-6 mg/kg and radiation at a wavelength 753 nm and fluence 200 J/cm was delivered through optical fibres, embedded into prostate gland under TRUS guidance. Three treatment plans: Targeted, subtotal or hemiablation were realised upon gadolinium-enhanced MRI guidance. The results of study revealed that VTP mediated with padeliporfin with photosensitizer dose 4 mg and radiation fluence 200 J/cm with LDI = 1 is an effective and safe method of LCaP trearment. In patients treated according to this protocol, a maximal therapeutic effect (no gadolinium sequestration in 95% of PTV) was observed by MRI at a week after VTP session and a negative biopsy rate was indicated as 83% at 26 wk after VTP procedure. At the same time, average scores of IPSS and quality of life revealed statistically significant improvement comparing to baseline whereas IIEF-5 score did not significantly change. In contrast, patients receiving padeliporfin at a dose of 2 mg/kg revealed no significant therapeutic effect, while in the men receiving photosensitizer at a dose of 6 mg/kg, necrotic areas in adjacent organs were indicated, although no clinical consequences of this event were reported[74].

Eymerit-Morin *et al*[89] investigated histological changes in biopsies of 56 patients with LPCa, taken 6 mo after VTP mediated with padeliporfin infusion and low-energy laser radiation delivered to the tumour environment by optic fibres inserted through the transperineal route. In 53 patients, sharply demarcated hyaline fibrotic scars, with rare atrophic glands, in the some cases reduced to corpora amylacea surrounded by huge multinuclear macrophages, were detected. Mild chronic inflammation, hemosiderin, and coagulative necrosis were also shown. The residual cancer in a treated lobe of 17 patients, was always located outside the scar, most often close to the prostate capsule, and revealed no changes related to VTP. In contrast to radiotherapy or hormone therapy, interpretation of histological changes after padeliporfin-mediated VTP was easy. This modality caused complete ablation of carcinoma within the targeted tissue[89].

Steba Biotech[90] and Azzouzi *et al*[91] made a multi-centre, multi-arm, open-labeled, phase Ⅱ clinical trial, to estimate the optimal treatment conditions for accomplishment of prostate tumour ablation and to evaluate the therapeutic effects of VTP mediated with padeliporfin in 86 patients with LPCa. According to the treatment protocol, padeliporphin was administered intravenously at doses of 4 or 6 mg/kg and radiation at a wavelength of 753 nm and at fluence of 200 or 300 J/cm, was delivered through transperineal interstitial optical fibres. The fibres were positioned in the prostate gland under ultrasound imaging and tumour location was additionally established using MRI and transrectal biopsy. The number of fibres and the total light energy were adapted to each patient individually, based on a treatment planning proposed by treatment planning group[91]. Biopsy results, dynamic contrast-enhancement MRI at a week after treatment and analysis of the safety information revealed that 4 mg/kg padeliporfin and 200 J/cm radiation create the optimal treatment conditions for the VTP treatment of LPCa, leading to negative biopsies at 6 mo in > 80% of patients treated with this regimen. Moreover, this procedure was well tolerated by patients and showed early signs of efficiency for minimally invasive focal treatment of LPCa[90].

A prospective, multicentre, open-label, phase Ⅱ clinical trial was also completed, to establish the optimal photosensitizer concentration and radiation fluence for achievement of prostate ablation with use of padeliporfin-mediated VTP in men with early prostate cancer[92]. The efficiency criteria were histological evaluation of a 6-mo biopsy, and assessment of hypoperfusion volume using gadolinium-enhanced MRI at 7 d after treatment. Safety and health-related quality of life were also evaluated. The results of this trial were reported in the work of Moore *et al*[67]. In this trial, 40 patients suffering from low-risk prostate cancer received padeliporfin at a dose of 2, 4 or 6 mg/kg, following irradiation with 753 nm radiation at a fluence of 200 J/cm. Photosensitizer was administered intravenously in a 10 min infusion. Radiation was delivered using diffusing fibres positioned in the prostate gland upon TRUS guidance. To evaluate treatment results, MRI at 7 d after treatment was used. IPSS, IIEF-5 and adverse effects at 7 d, 1, 3 and 6 mo after VTP were also assessed. The biopsies guided with TRUS were collected at 6 mo. The three treatment plans for focal VTP therapy were applied: Whole gland ablation, hemiablation and bilateral quadrant ablation that targets a quarter of the prostate to spare the remainder of the untreated gland[20,67,92].

Maximal treatment effect (95% of the PTV) was observed by MRI at 7 d after illumination, in patients who received photosensitizer at a dose of 4 mg/kg, radiation fluence of 200 J/cm and whose LDI was higher than one. In the case of 12 men treated with these parameters, the negative biopsy rate was 83% at 6 mo, comparing to 45% determined for the subjects who received other drug doses (10 patients) or whose LDI was lower than one (16 patients). Both IPSS and IIEF-5 scores were not significantly different between baseline and 6 mo after VTP. As adverse events, only transient urinary inconveniences were reported by the patients. No cases of hypotension, which is an important problem in padoporfin-mediated VTP, were observed. These results suggest that VTP using padeliporfin at a dose of 4 mg/kg and irradiation with 753 nm radiation at a dose of 200 J/cm may be promising modality of the treatment of early prostate cancer leading in patients with LDI > 1 to necrosis 95% of the planned treatment volume and to negative biopsy rate at 6 mo of 83% men[57,67].

Phase Ⅱ/Ⅲ trials

The open-labelled, multicentre, 6-mo phase Ⅱ/Ⅲ clinical trial with an additional follow-up at 12 mo, was initiated, to establish efficiency and tolerability of the padoporfin-mediated VTP treatment of prostatic carcinoma. According to treatment protocol padoporfin was administered to patients at a dose of 2 mg/kg in the intravenous infusion, while laser radiation at a wavelength of 763 nm was delivered through optical fibres inserted through the perineum to the prostatic lobes. Patients who are eligible to participate in this trial presented with clinically diagnosed positive biopsies diagnosed after external radiotherapy or temporary brachytherapy, and with increasing PSA concentrations on three subsequent measurements after radiotherapy. This study has been terminated because of sponsor decision to develop padeliporfin as a safer and more efficient candidate for therapeutic applications[93].

During Ⅱ and Ⅲ Phase clinical trials, the standardised procedure of PCa treatment using padeliporfin-mediated VTP, was drawn up. General anaesthesia was necessary to achieve complete immobility of patients during the whole procedure and at the same time to keep safety and efficiency of treatment. The prostate and the adjacent structures were visualised by the biplane TRUS probe. For installation of optical fibres, the transparent fibre insertion catheters (FIC) were situated into the prostate transperineally through the template using the TRUS scan system, according to the treatment guidance provided by the software TOOGUIDE®. The optical fibres were calibrated to adjust the radiation power within ± 5 mW. The positions of these fibres defined a precisely targeted treatment area. With the optimal treatment conditions each centimetre of fibre induced 0.8-1 cm^3 of necrosis with more than 90% of necrosis of the targeted volume. The LDI was above one to assure favorable condition irradiation, better than in the case of hemiablation procedure. When all FICs and optical fibres were in position, the light of the room was dimmed and the patient was entirely protected from light exposure. The only exposed zone was the perineum. The infusion of photosensitizer was administered using opaque syringe and line.

Patients received padeliporfin at a dose of 4 mg/kg in a single, 10 min intravenous infusion and prostate glands were continuously irradiated through diffusing

Table 2 Vascular targeted photodynamic therapy using padoporfin and padeliporfin in the treatment of localised prostate cancer - clinical trials

Phase	No. of patients	Photosensitizer	Radiation	Ref.
I	10	Padoporfin, 0.1-2 mg/kg (0.1, 0.25, 1 and 2 mg/kg)	763 nm, 100-360 J/cm	Weersink *et al*[83]
I	24	Padoporfin, 0.1-2 mg/kg	763 nm, 100, 230 and 360 J/cm	Trachtenberg *et al*[56]
I / II	15	Padoporfin, 0.1-2 mg/kg	763 nm, 100 J/cm	Gertner *et al*[84]
I / II	34	Padoporfin, 2 mg/kg	763 nm, 100-300 J/cm	Arumainayagam *et al*[86]
I / II	30	Padeliporfin, 2, 4 and 6 mg/kg	753 nm, 200 and 300 J/cm	https://clinicaltrials.gov/ct2/show/NCT00946881[87]
II	28	Padoporfin, 2 mg/kg	763 nm, 0.1-1000 J/cm	Trachtenberg *et al*[57]
II	40	Padeliporfin, 2, 4 and 6 mg/kg	753 nm, 200 J/cm	Arumainayagam *et al*[72]
II	40	Padeliporfin, 2-6 mg/kg	753 nm, 200 J/cm	Quoraishi *et al*[74]
II	85	Padeliporfin, 4 mg/kg	753 nm, 200 J/cm	Azzouzi *et al*[88]
II	56	Padeliporfin, 4 mg/kg	753 nm, 200 J/cm	Eymerit-Morin *et al*[89]
II	86	Padeliporfin, 4 and 6 mg/kg	753 nm, 200 and 300 J/cm	https://clinicaltrials.gov/ct2/show/-NCT00975429[90]
II	117	Padeliporfin, 4 mg/kg	753 nm, 200 J/cm	Azzouzi *et al*[62]
II	40	Padeliporfin, 2, 4 and 6 mg/kg	753 nm, 200 J/cm	Moore *et al*[67]
II	40	Padeliporfin, 2, 4 and 6 mg/kg	753 nm, 200 and 300 J/cm	https://www.clinicaltrials.gov/ct2/show/NCT00707356[92]
II / III	86	Padeliporfin, 4 mg/kg	753 nm, 200 J/cm	Azzouzi *et al*[91]
II / III	16	Padoporfin, 2 mg/kg	763 nm, no information on radiation fluence	https://www.clinicaltrials.gov/ct2/show/-NCT00312442[93]
II / III	1	Padeliporfin, 4 mg/kg	753 nm, 200 J/cm	Azzouzi *et al*[94]
II / III	19	Padeliporfin, 4 and 6 mg/kg	753 nm, 200 and 300 J/cm	Lebdai *et al*[95]
III	81	Padeliporfin, 4 mg/kg	753 nm, 200 J/cm	https://clinicaltrials.gov/ct2/show/-NCT01875393[96]
III	400	Padeliporfin, 4 mg/kg	753 nm, 200 J/cm	https://clinicaltrials.gov/show/-NCT01310894[97]

optical fibres with a 753 nm radiation at a radiation dose of 200 J/cm, delivered by a multichannel diode laser. The irradiation started immediately after the end of the infusion and lasted 22 min and 15 s, to coincide with the maximal plasma concentration of padeliporphin. The total duration time of the full procedure was 1.5 to 2 h, depending on the volume of the targeted area and the number of optical fibres to be placed[68,88,94].

Lebdai *et al*[95] evaluated safety, efficiency and feasibility of salvage radical prostatectomy (RP) after padeliporfin-mediated VTP using results obtained for 19 patients from France during II phase (NCT00707356 and NCT00975429) and III phase (NCT01310894) clinical trials. The median of operation time, of hospital stay and of delay between VTP and RP were 150 min, 7 d and 17 mo, respectively. During operation, median blood loss was 150 mL, and median PSA concentrations before and after operation were 6.30 and 0.02 ng/mL, respectively. No perioperative mortality has been reported, and only 3 patients revealed complications such as pelvic hematoma, or superficial wound infection. Several patients revealed erectile dysfunctions before or after RP. Positive margins were significantly associated with bilateral VTP. Six patients underwent complementary radiotherapy. These outcomes suggest that salvage RP after VTP treatment is a safe, efficient and feasible method for treatment of locally recurrent PCa. However, to confirm this supposition, long-term studies are necessary[95].

Phase III trials

For evaluation of efficiency and safety of padeliporfin-mediated VTP in treatment of LPCa as well as for assessment of patient quality of life after this treatment, the interventional phase III clinical trial has been developed. For this study, 81 patients from Mexico, Panama and Peru have been recruited. Padeliporfin was administered at a dose of 4 mg/kg in 10 min infusion, following laser irradiation at a wavelength of 753 nm and radiation fluence of 200 J/cm, delivered transperineally through optical fibres embedded into the prostate under ultrasound imaging. The follow-up was conducted 12 mo after irradiation, assessing quality of life, urinary and erectile functions. PSA concentrations were determined at 3, 6 and 12 mo after application of VTP and clinical efficiency of this method was evaluated at 1, 3, 6 and 12 mo after its application. The results of this trial are currently completed[96].

The multicentre, randomised controlled, open label phase III clinical study to compare safety and efficiency of padeliporfin-mediated VTP with active surveillance in treatment of localised prostate cancer was also initiated. This study will include 400 patients, from which a half will be treated with active surveillance and the other half with VTP mediated with padeliporfine. The procedure will be the same as in the trial described above. This trial is not yet enrolling participants[97].

The main characteristics of described clinical trials are summarized in Table 2.

CONCLUSION

VTP holds promise as a novel strategy of the focal treatment of LPCa. This treatment modality is convenient to perform, minimally invasive and do not need long anaesthesia (usually about 2 h), therefore it can be conduct in ambulatory conditions. Intraperitoneal irradiation enables changing radiation parameters during

VTP session, to target tumour foci more precisely and to avoid the risk of adverse effects on parts of prostate which are not seized by cancer lesions as well as on tissues and organs surrounding prostate gland[5,70].

Although VTP is not yet the standard strategy for organ confined PCa, it is the therapeutic approach with the most important future potential. To make this strategy a standard element of PCa therapy, the new VTP protocols, founded on the real-time feedback and rules-based approach of treatment parameters, are necessary[44,97]. Multiparametric MRI with gadolinium contrast may be suitable for detection and characterisation of therapy progress during VTP treatment[98-101]. Intraoperative contrast enhanced high-intensity focused ultrasound may also play a role in VTP monitoring, however this imaging technique is not exactly real-time by its nature[102,103]. In order, rules-based approach would involve specific fibre density, or specific limits at different prostate boundaries[77]. As some authors indicate the low costs of photochemotherapeutic methods as their advantage over other strategies of prostate cancer therapies[17], a systematic cost-effectiveness analysis for VTP application in PCa treatment is indispensable.

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REVIEW

Updated overview of current biomarkers in head and neck carcinoma

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Abstract

Squamous cell cancer is the most common type of malignancy arising from the epithelial cells of the head and neck region. Head and neck squamous cell carcinoma (HNSCC) is one of the predominant causes of cancer related casualties worldwide. Overall prognosis in this disease has improved to some extent with the advancements in therapeutic modalities but detection of primary tumor at its initial stage and prevention of relapse are the major targets to be achieved for further improvement in terms of survival rate of patients. Latest achievements in basic research regarding molecular characterization of the disease has helped in better perception of the molecular mechanisms involved in HNSCC progression and also in recognizing and targeting various molecular biomarkers associated with HNSCC. In the present article, we review the information regarding latest and potential biomarkers for the early detection of HNSCC. A detailed molecular characterization, ultimately, is likely to improve the development of new therapeutic strategies, potentially relevant to diagnosis and prognosis of head and neck cancers. The need for more accurate and timely disease prediction has generated enormous research interests in this field.

Key words: Head and neck squamous cell carcinoma; Early detection; Prognosis; Biomarkers; Molecular level

Core tip: Early detection of head and neck squamous cell carcinoma is vital in improving the overall survival and prognosis. It can be achieved by use of latest biomarkers. With advancement in knowledge of molecular characteristics of this disease, various biomarkers acting at molecular level have been identified. This review compiles information regarding the potential players in this field.

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INTRODUCTION

The term head and neck carcinoma encompasses all malignancies arising in the nasal and oral cavities, pharynx, larynx and the paranasal sinuses. Majority of these (approximately 95%) epithelial cancers are squamous cell carcinomas[1]. Smoking and alcoholism are two well known predisposing factors[2]. Head and neck squamous cell carcinoma (HNSCC) is reported to be the sixth common cause of cancer mortality throughout the world[2].

There is no significant improvement in the mortality rates even with continuous research and trials in the field of diagnostics and therapeutics[3]. As compared to other cancers like breast, cervix and colorectal, the five-year survival rate of HNSCC after diagnosis is significantly lower[4,5]. The reason for this could be failure in early diagnosis and insufficient effectiveness of therapeutic modalities[6,7]. The predominant cause of mortality in HNSCC is regional and/or distant metastatic spreading of tumor cells from primary site[8]. Therefore, the vital area in the treatment of head and neck cancers is ability to diagnose it at an early stage.

EARLY DIAGNOSIS OF HNSCC

Till date only one third cases of HNSCC are being diagnosed at an early stage and rest land up with an advanced disease in the United States[9,10]. The major reason put forward for this trend include a lack of appropriate screening biomarkers[11]. The treatment of neoplasia is most effective in its early stage when the tumor size at primary site is lowest with least lymphatic and hematogenous spread. Therefore, early diagnosis and intervention is of utmost significance in the treatment of HNSCC. Here comes the role of biomarkers. Biomarkers may be analyzed in the tissue itself, plasma or other body fluids like saliva in case of HNSCC. The drawback of biomarkers may include lack of specificity and sensitivity but these may prove to be essential tools in timely diagnosis of the disease[12]. A variety of biomarkers have been reported in literature with a promising potential but these are still in the need of clinical validation. In this article, we present a review of different biomarkers which may be utilized in early diagnosis and timely decision-making for intervention in patients of HNSCC.

ALTERATION IN EXPRESSION OF CHEMOKINE RECEPTORS

Recently, the importance of chemokines and their cognate receptors in head and neck cancers is being reported by increasing number of studies.

CXC chemokine receptor 2

In the squamous cell carcinoma of the larynx, the expression of CXC chemokine receptor 2 (CXCR2) has been observed to be substantially higher in tumor tissue than that in the paraneoplastic tissue. The increased expression has been reported to be significantly related with lymph node metastasis, histological grade and 5-year survival of these patients. Thus, expression of CXCR2 can be considered as a potent prognostic marker for laryngeal squamous cell carcinoma[13].

CXCR4

The importance of CXCR4 in tumour progression and organ-specific metastasis in patients with HNSCC has been reported by a number of authors[14,15]. Wang et al studied the expression of CXCR4 in nasopharyngeal carcinoma tissues and found an increased CXCR4 expression in tumor tissues. Besides this, they also suggested that the increased expression of CXCR4 may be correlated with increased metastatic rates and poor overall survival of the patients[16]. This finding was consistent with another study which also reported significantly elevated CXCR4 mRNA in HNSCC tissues as compared to paraneoplastic tissues and that the increased expression was associated with increased risk of lymph node metastasis and distant metastasis[17]. Therefore, CXCR4 expression can also be used as a marker to predict prognosis and metastasis in patients with HNSCC.

CC chemokine receptor 7

CC chemokine receptor 7 (CCR7) is another CC chemokine receptor, which has been demonstrated to play a significant role in the migration of activated dendritic cells to regional lymph nodes. Its expression has also been reported to be elevated in HNSCC tumor tissues as compared to paraneoplastic tissues. Furthermore, the elevated expression of CCR7 has been found to correlate with lymph node metastasis and tumor tissue histological differentiation status[17]. Similar findings have been reported by another study which analyzed the expression of CCR7 in primary and metastatic tumor cell lines and also in biopsy material from both primary and metastatic lesions. They reported that CCR7 expression was increased in metastatic cells and tissues[18]. On the basis of these reports, an important role may be conferred to CCR7 in predicting the metastasis and prognosis in HNSCC patients.

HUMAN PAPILLOMA VIRUS

Human papilloma virus (HPV), especially HPV16, is considered one of the causing factors for HNSCC. HPV DNA has been found in 15% to 25% of HNSCC and the association differs depending on the site of the tumor[19]. HPV DNA is detected in 45%-67% of cases of cancers of the tonsil, in 13%-25% of hypopharyngeal cancer, in 12%-18% of the cancers of oral cavity and in 3%-7% of carcinoma larynx and it may be associated with prognosis of disease, especially in tonsillar cancers[20]. There are reports in literature suggesting that HNSCC with HPV has a favorable prognosis and that, it in fact, is a distinct clinicopathological entity[21]. HPV16 and HPV18

are considered to be the high risk HPVs, which produce E6 and E7 oncoproteins, implicated in transformation of cell and altering the control of cell cycle. Oncoprotein E7 binds to and induces the proteolysis of pRb while E6 inactivates p53 by accelerating its ubiquitin mediated degradation[22]. Thus, HPV DNA may act as a diagnostic and prognostic marker in patients of HNSCC.

It is of interest to know that adding $p16^{INK4A}$ immunostaining to HPV DNA detection may prove to be very useful in diagnosing HPV-related oral squamous cell carcinoma and it has been observed that HPV(+) and $p16^{INK4A}$(+) types of tumors have better prognosis[23]. As reported by Danish Head and Neck Cancer Group 5 trial, $p16^{INK4A}$(+) tumors appeared to be associated more strongly with poor histopathologic differentiation as compared to the $p16^{INK4A}$(-) ones, but the difference was not statistically significant, indicating that $p16^{INK4A}$ alone is not an adequate marker[24]. In the study of panitumamub efficacy in patients with recurrent and/or metastatic head and neck cancer (SPECTRUM), the authors reported that the $p16^{INK4A}$ status of the tumor might have significant bearings in designing future trials in cases of recurrent or metastatic HNSCC[25].

MICROSATELLITE INSTABILITY

Microsatellite instability (MSI) may be analyzed using different markers. Researchers have detected loss of heterozygosity (LOH) in tumor cell derived DNA (deoxyribonucleic acid) from mouth washing or lesion brushing samples in patients with T2N0M0 and T1N0M0 tumors[26]. MSI analysis in tumor cell DNA is of value in detection of pre-malignant conditions like erythroplakia and leukoplakia[1]. It has also been reported that LOH of 9p21 may be an initial event in HNSCC and may be associated with preneoplastic lesions as well as 30% of cases of squamous cell carcinoma[27]. Loss of chromosomal region 9p21 is seen in > 70% of cases, making it the most frequent genetic alteration seen in squamous cell dysplasia and HNSCC[27,28]. Some of the studies in which MSI was analyzed using different set of markers in patients with HNSCC have also reported MSI in 12.5%-35% of the cases while microsatellite alteration rate was detected to be 75%-95%[29-31]. Instability frequency has been reported to be related to the repeat unit length and overall size of the short tandem repeat (STR) affecting the probability of error during DNA replication. STR characteristics vary in different populations and those with longer average repeat size are more prone to instability than the ones having smaller repeat size[31].

MSI analyses have the disadvantage of lack of uniformity in selection of different methods or the type and number of markers evaluated[32]. A standard approach is yet to be developed for this marker to be useful as an early diagnostic marker in HNSCC patients.

METHYLATION

Gene activation due to hypermethylation of cytosine-phosphate-guanine (CpG)-rich promoter regions has been reported in early stages of HNSCC[30]. A specificity of 96% in salivary specimens for methylation specific polymerase chain reaction has been reported for detection of HNSCC[33]. Whereas it was observed to be 90% in salivary samples and 72% for serum samples in yet another study[34]. The lower rate of promoter hypermethylation may be due to dilution with normal, non-methylated DNA from normal mucosal areas[34]. It has also been reported that promoter hypermethylation may be associated with age and ethnicity of the patient or with history of chronic tobacco or alcohol consumption[35,36].

The disadvantages of methylation markers include lack of sensitivity, specificity, complexity and inconvenience in HNSCC detection in body fluids.

METALLOPROTEINASES

These include a large number of zinc and calcium dependent endopeptidases. These enzymes are implicated in extracellular matrix degradation leading to spread of the tumor cells out of the tissue of origin[37-39]. Besides migration of tumor cells, metalloproteinases (MMPs) play a significant role in providing a microenvironment conducive for the growth and angiogenesis of tumors. These also help in cellular differentiation, proliferation and apoptosis in tumor tissues[38]. Several types of MMPs, *e.g.,* MMP-1, the gelatinases (MMP-2 and MMP-9) and the stromelysins (MMP-3 and MMP-10) play a role in tissue invasion by cancer cells and metastasis[40,41]. Elevated levels of MMP-2 or MMP-9 have been observed in many types of cancers including HNSCC, lung, breast, colorectal and ovarian carcinoma indicating an association with tumor progression[42-45].

In HNSCC, patients have been found to have increased levels of MMP-3, MMP-8 and MMP-9[46] while MMP-1 and MMP-10 have been reported to be useful for detection of cancer of oral cavity and gingiva[47]. In another study, MMP-9 has been reported to be able to detect stage Ⅰ HNSCC disease with 80% positivity[48]. The disadvantage with MMP-9 lies in its poor specificity to discriminate cancer with benign disease[49].

INTERLEUKINS

Interleukin (IL)-6 and IL-8 have been linked with tumor progression and metastasis along with playing a role in the process of carcinogenesis[12]. IL-8 holds potential for acting as an early biomarker in salivary samples while IL-6 in serum samples for detection of oral cavity or oropharynx squamous cell carcinoma (OSCC)[50,51]. In some other studies, increased levels of IL-6 and IL-8 have been reported in a variety of specimens like cell line supernatants, tumor tissues and serum of patients with HNSCC[52,53].

Zimmermann *et al*[54] reported that four mRNAs (OAZ, SAT, IL-8 and IL-1β) in salivary samples have a collective sensitivity and specificity of 91% in detection of cancer of oral cavity. On the other hand, the levels

of salivary IL-8 were found to be raised in patients of OSCC as compared to controls but the difference was not statistically significant[55]. Thus, further studies are required to establish the sensitivity and specificity of IL-8 and IL-6 as biomarkers in patients of OSCC.

MICRO RNA

These are small non-coding RNA (ribonucleic acid) sequences playing a role in regulation of gene expression affecting a variety of physiological processes[56]. miRNAs, by virtue of their vast range of consequences may act both as oncogenes and tumor supressor genes[57]. In many types of cancers, dysregulation of genes for miRNAs has been reported and these can be used for detection and classification of different solid tumors[58]. The change in micro RNAs (miRNAs) in cancer cells as compared to normal cells has been reported to be many folds than the extent of change in mRNA[59].

It has been proposed that miR-106b-25 cluster and miR-375 may be involved in development and progression of HNSCC and that miR-451 could act as a prospective prognostic marker for recurrence in HNSCC patients. The same authors also observed one third of the miRNAs to be dysregulated in HNSCC[60]. Park *et al*[61] reported significantly lower levels of miR-125a and miR-200a in the saliva of OSCC patients as compared to controls. miR-205 has been found to have a variable expression in a number of tumor cells and, particularly, to be highly overexpressed in HNSCC cell lines and may prove helpful in detecting occult metastatic tumor deposits[62,63]. Deregulation of miR-138 has been commonly found in HNSCC and other types of cancer. A number of functional targets for miR-38 have been reported which include genes involved in initiation and progression of HNSCC[64]. It has also been demonstrated that restoration of transfected miR-34a mimics significantly inhibits the capability for epithelial-mesenchymal transition of cancer stem cell-phenotype and functionally decreases clonogenic and invasive capability in HNSCC cell lines[65].

Micro RNA biomarkers are superior to their mRNA counterparts. Because of their robust profiling and better stability in routine clinical samples, they may prove to be more suitable for analysis in some tissue samples[64]. Thus, miRNA may prove to be promising early biomarkers in detection of HNSCC but further research is needed to substantiate their role as screening tools.

MELANOMA-ASSOCIATED GENE

Melanoma-associated gene (MAGE) participates in the process of carcinogenesis by suppressing apoptosis[66]. Other similar tumor-specific shared antigen families like G antigen gene, B melanoma antigen gene and L antigen family 3 gene have been categorized at molecular levels[67-69]. These antigens, usually peptides in nature, may be significantly associated with tumor immunology as their expression has been found specific to tumor cells, *e.g.,* HNSCC, melanoma, carcinoma ovary, bladder cancer, carcinoma lung and colorectal cancer[70-73]. Expression of MAGE A3 and A4 has been found to be positive in early invasive carcinoma (by excisional biopsy) where brush and incisional biopsy was negative in a suspicious looking leukoplakic lesion[74]. Expression of MAGE has also been shown in the sputum samples of patients with HNSCC[75]. Therefore, it may be used as an early biomarker for HNSCC detection as it has not been observed to be expressed in normal healthy tissues with exception of testis[76]. Other studies have also reported 85.5%-90% expression rate of MAGE in HNSCC tissue[77,78]. It may help in initiating target specific immunotherapy in these patients[79]. According to a recent report by Lee *et al*[80] expression of MAGE-A1-6 in sputum predicts poor oncologic outcome in patients with squamous cell carcinoma of the larynx and hypopharynx. MAGE-A expression has been reported to be associated with poorer five year survival rate, thus, indicating its potential as a prognostic marker also[81].

CENTROSOME ABNORMALITIES

Centrosome abnormalities have also been observed in HNSCC. It has been reported that 17 out of 18 tumor samples analyzed from patients with HNSCC demonstrated centrosome hyperamplification. Based on these findings, it has been suggested by authors that centrosomal hyperamplification could be used as a marker for HNSCC[82]. Furthermore, the p53 suppressor gene, the most commonly mutated gene found in human cancers, has been reported to correlate with centrosome hyperamplification in HNSCC. Centrosome hyperamplification is either observed in tumors with mutated p53 or in tumours that retain wild type p53 but with an overexpressed Mdm2, an oncogene which is responsible for inhibiting the transactivation function of p53[83]. Increased frequency of centrosomal abnormalities has also been seen in OSCC in cells with spindle checkpoint protein CDC20 overexpression[84]. This may be because of the fact that in cancer cells, genes that encode for proteins involved in mitotic checkpoints/mitotic regulations are generally found mutated or overexpressed.

ACTIN AND MYOSIN

These are cytoskeletal proteins responsible for cell motility and invasion which are important components of epithelial tumorigenesis[85]. Increased expression of actin and myosin has been observed in exfoliated cells present in soluble saliva in patients with malignancy as compared to those with pre-cancerous lesions[86]. Increased actin isoforms have been observed in invasive basal cell carcinoma[87], squamous cell carcinoma of cervix[88] and esophagus[89] and invasive OSCC[90]. Increase in myosin abundance has been observed in proteomics of tissue from OSCC region[91]. However, Turhani

et al[92] have reported a lesser expression of myosin light chain in HNSCC contradicting the existing findings.

Thus, actin and myosin need rigorous research with larger sample groups including issues like sensitivity and specificity for their establishment as HNSCC biomarkers.

CYTOKERATINS

Cytokeratins (CKs) are one of the major components of intracellular filament network found in different tissues[93]. CKs are expressed in a number of combinations depending on the type of epithelial cell of origin[94]. They are further divided into two subtypes (Ⅰ and Ⅱ) that are generally coexpressed[95,96]. These are found to be overexpressed in OSCC tissue as compared to normal mucosa[93]. Overexpression of cytokeratins has been related with tumor progression and prognosis[97]. Constitutive expression of cytokeratin-17 (CK-17) in the lungs is only found in the normal basal cells[98]. It is now emerging as a tissue-specific immunohistochemical biomarker in squamous cell carcinoma of larynx[99]. CK-17 mRNA overexpression has been demonstrated in OSCC by few authors[96,99,100]. These studies were mainly performed in cancer tissue and not in saliva or serum samples. Increased expression of CK-17 has been demonstrated in respiratory syncitial virus infected epithelial cells also[95].

As CK-6 and CK-16 are found to be constitutively expressed in mucosal stratified squamous epithelia, they may be ragarded as markers of cellular hyperproliferation. CK-6 may also be considered as an additional squamous differentiation biomarker in poorly-differentiated cancers. Though CK-17 was also detected in most of the cases, its expression is not found to be uniform[101].

These markers need stringent workup with larger samples, including body fluids and also on issues regarding its specificity before their validation as biomarkers for HNSCC.

p53

It is the most frequently studied molecular marker in HNSCC[102]. The p53 pathway is activated when cells become old or damaged. The p53, a 53 kd protein, may then arrest cell cycle for DNA to be repaired or lead to apoptosis if damage is irreparable[103]. Alteration in function of p53 may be seen as a result of mutation or sequestration by other cellular proteins. Mutations of p53 gene are the most commonly encountered mutations in carcinomas including HNSCC[102,104]. p53 is associated with maintenance of cellular integrity and is regarded as guardian of genome[105]. Mutations in p53 in HNSCC patients have been reported by a number of researchers with an expression range of 50%-60% of the tumor cells[103,105-108]. Its expression can be conveniently studied with immunohistochemistry techniques for detection of cancers but complete role of p53 in pathogenesis of HNSCC is still not clear[109,110]. Survival rate has also been reported to be higher in p53 negative patients as compared to those who are positive for p53 mutations[102]. Thus, this marker has a fair potential for diagnostic and prognostic use in patients with HNSCC. Another interesting finding is that HPV infection rarely coexists with p53 mutation as both of them can independently lead to p53 inactivation implicated in HNSCC tumor[111].

EUKARYOTIC TRANSLATION FACTOR 4E

It is a protein involved in the initiation of protein synthesis[112]. Overexpression of eukaryotic translation factor 4E (eIF4E) has been found associated with different stages of carcinogenesis including metastasis. It is related with transformation of fibroblasts and primary epithelial cells[113,114]. Overexpression of this protein in mice has been found to be associated with a number of malignancies like lymphomas, angiosarcomas, hepatomas and carcinoma lung[115]. An expression of 100% in HNSCC has been reported in some studies[116,117]. Overexpression of eIF4E in cancers like breast, bladder, lung and HNSCC has been found to correlate with an increased risk of disease progression and poor prognosis[113,118-121]. Another study has reported overexpression of eIF4E in tumor free surgical margins to be related to loco-regional recurrence in patients of HNSCC[122].

Therefore, eIF4E may prove to be a significant independent prognostic predictor in terms of recurrence and survival in patients of HNSCC.

LOSS OF FUNCTION OF DNA REPAIR GENES

Effective DNA repair may be considered as a major determinant of cancer-free survival. Various mutations in DNA repair genes, especially, of the nucleotide excision repair (NER) group (XP genes in xeroderma pigmentosum patients), DNA crosslink repair (Fanconi anemia genes) mutations affecting the mismatch repair genes, and a number of others are the cause of several hereditary cancerous syndromes[123]. The dominant moderator of mismatch repair in HNSCC is promoter hypermethylation rather than direct mutation[124]. There is also limited data in HNSCC demonstrating a link between poly-(ADP-Ribose) polymerase overexpression and cisplatin resistance suggesting a possible role for chemoresistant tumours. Hyperphosphorylation of replication protein A, a single-strand DNA binding protein, that is, integral to HR, has also been implicated as a mechanism for cisplatin resistance in HNSCC cell lines[124]. Multiple (5-7) risk NER genotypes have been associated with a 2.4-fold increased relative risk of second primary HNSCC[124]. The inactivation of these DNA repair genes may be linked to carcinogenesis by decreasing genomic stability and producing certain genetic alterations[125] (Table 1).

Table 1 Comparison of different biomarkers of head neck squamous cell carcinoma

Marker	Mechanism	Type of specimen	Role	Limitations
Chemokine receptors	Increased expression in tumor tissue	Biopsy specimen	Prognosis and metastasis	Clinical validation by further research required
HPV	DNA associated HNSCC, oncoprotein production	Tumor tissue	Diagnosis and prognosis	Lack of sensitivity and specificity
MSI	LOH in tumor derived DNA	Mouth washings/lesion brushings	Detection of pre-malignant lesion	Lack of uniformity of method
Methylation markers	Gene inactivation following hypermethylation in promoter region	Saliva/serum	Early detection	Lack of sensitivity/specificity, complex methodology
MMPs	Provide conducive microenvironment for tumor growth, degrade ECM promoting tumor migration	Tumor tissue/saliva	Early detection	Poor specificity
Interleukins	Participate in process of tumor growth and metastasis	Tumor tissue/cell line supernatants/saliva/serum	Early and convenient biomarker	Lack of sensitivity and specificity
miRNA	Role in regulation of gene expression	Tumor tissue/saliva	Early detection	Clinical validation required
MAGE	Suppresses apoptosis	Biopsy specimen/saliva	Prognosis and in selecting targeted immunotherapy	Clinical validation required
Centrosome abnormalities	Mutation due to hyperamplification	Tumor tissue	Early detection	Further research required to understand molecular mechanism
Actin and myosin	Increased expression leading to greater invasiveness	Tumor tissue/saliva	Early detection	Lack of sensitivity and specificity
Cytokeratins	Over-expression associated with tumor progression	Tumor tissue/saliva/serum	Early detection and prognosis	Clinical validation required
p53	Mutation affects apoptosis/repair of malignant cells	Tumor tissue	Diagnosis, prognosis, convenient marker	Complete role in HNSCC yet to be deciphered
eIF4E	Overexpression associated with tranformation of fibroblasts and epithelial cells	Tumor tissue	Prognostic indicator	Lack of sensitivity and specificity

HNSCC: Head and neck squamous cell carcinoma; MSI: Microsatellite instability; LOH: Loss of heterozygosity; MMP: Metalloproteinase; ECM: Extracellular matrix; MAGE: Melanoma-associated gene; HPV: Human papilloma virus; miRNA: Micro RNA; eIF4E: Eukaryotic translation factor 4E.

ROLE OF IMAGING BIOMARKES

Besides biochemical and pathological biomarkers, a significant role is being played by imaging biomarkers in the early detection of head and neck oncology. To go into the details of these biomarkers is beyond the scope of the present article, but these are proving to be vital in initial staging, treatment planning, monitoring and follow-up of the patients with HNSCC non-invasively. ^{18}F-fluoro-2-deoxyglucose positron emission tomography/computerized tomography (PET/CT) proves to be more sensitive and specific as compared to magnetic resonance imaging (MRI) or CT alone[126,127]. Recently introduced regional PET/Gd (gadolinium-enhanced T1-weighted)-MRI combined with whole-body PET/MRI appears to be quite promising in detecting early lesions[128]. There is a need for further refinement and a concerted approach regarding imaging and molecular biomarkers for HNSCC which may help in early detection, targeted therapy and improved monitoring.

CONCLUSION

Understanding the molecular mechanisms of HNSCC is important to identify its biomarkers. Finding genetic alterations can lead to early detection of the disease. These can be detected in tumor tissue, saliva/body fluids washing the affected tissue or in the serum. A variety of molecular markers have been explained in literature. There may be a tremendous role of these markers in affecting the outcome of the disease by aiding in timely diagnosis and even in selecting specific therapy. Many of these have already shown their potential in this field like interleukins, MAGE, MSI, *etc.*, but still there are issues of specificity, sensitivity and clinical validation with some of these. With more standardised and uniform platform for sample selection, processing and data analysis along with stringent workup of the cases, these biomarkers may prove to be indispensable investigative tools in patients with HNSCC and may even help in better understanding of the pathogenesis of the disease. Thus, there is a strong hope that these molecular biomarkers or patterns of markers, alone or in co-ordination with imaging markers, could, in the future, be utilized for early detection of HNSCC, tumor metastasis and may aid in determining the best therapeutic modality for patient care.

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MINIREVIEWS

Cutaneous perivascular epithelioid cell tumors: A review on an infrequent neoplasm

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Abstract

"Perivascular epithelioid cutaneous" cell tumors (PEComa) are a family of mesenchymal tumors with shared microscopic and immunohistochemical properties: They exhibit both smooth muscle cell and melanocytic differentiation. Non-neoplastic counterpart of PEComa's cells are unknown, as well as the relationship between extracutaneous PEComa and primary cutaneous ones. We will review the clinical setting, histopathologic features, chromosomal abnormalities, differential diagnosis and treatment options for cutaneous PEComa.

Key words: Perivascular epithelioid cell tumor; Skin; Cutaneous perivascular epithelioid cell tumors; Clear cell myomelanocytic tumor

Core tip: We provide a comprehensive review of a rare neoplasm, cutaneous perivascular epithelioid cell tumor.

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INTRODUCTION

"Perivascular epithelioid cutaneous" cell tumors (PEComa) are a family of mesenchymal tumors with shared microscopic and immunohistochemical properties (they exhibit both smooth muscle cell and melanocytic

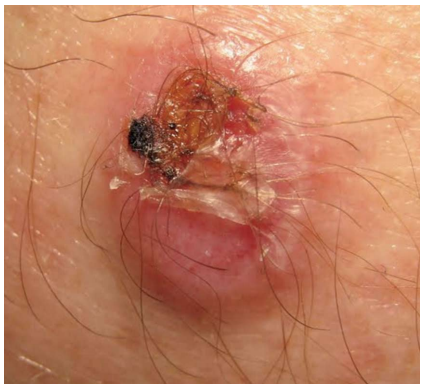

Figure 1 Pinky plaque with well-defined edges and a centrally located crust.

differentiation)[1].

This term, PEComa, introduced by Zamboni *et al*[2], includes a group of tumors with distinctive perivascular epithelioid cells such as angyomiolipomas, lymphangiomyomatosis, clear cell sugar tumor of the lung and the so-called PEComa, that have been described in various organs and tissues, including the skin[3-13]. In any case, PEComa are exceedingly rare; have been described in the pancreas[2], pelvic cavity[14], uterus[15], prostate[16], urinary bladder[17], digestive tract[18], vulva[18], heart[18], trachea[19], lymph node[7], breast[20], bone[21] and soft tissues[22]. Moreover, tumors fitting the definition of PEComa have been reported under different names, including "clear cell myomelanocytic tumor", "abdominopelvic sarcoma of perivascular epithelioid cells" and "primary extrapulmonary sugar tumor"[13].

"PEComa"

First "legitimate" cutaneous PEComa was reported by Mentzel *et al*[9] as an abstract. After that, several other reports appeared, as well as the first series of cutaneous PEComa[9].

The most characteristic histopathologic feature of these neoplasms is that they are composed of epithelioid cells with a clear or granular cytoplasm that tend to be arranged in perivascular fashion[1].

Normal counterpart of PEComa's cells is unknown, but there are several hypotheses including: (1) a differentiation line close to undifferentiated cells of the neural crest; (2) a myoblastic origin along with a molecular alteration that led to a melanogenesis activation; or (3) as a third option, a pericytic cell origin. Furthermore, the relationship between extracutaneous PEComa and primary cutaneous ones remains uncertain[23].

CLINICAL FEATURES

PEComa, as stated previously, are rare tumors, preferably located in subcutaneous soft tissues in the female genital tract or in the thorax (Figure 1). Cutaneous ones account for just 8% of cases, located mostly on the lower leg and, less commonly, on the forearm or the back. They usually behave in a benign fashion[8], although malignant examples have also been reported[7]. They typically appear in middle-aged adult females[24]. In our review of literature, we have found described 34 "legitimate" primary cutaneous PEComa[23]. Some of these neoplasms may be associated with tuberous sclerosis complex[4] but cutaneous lesions are mostly solitary lesions with no other associated anomalies[24].

HISTOPATHOLOGIC CHARACTERISTICS

Cutaneous PEComa presents usually as a well-demarcated dermal lesion that can extend to subcutis, composed of epithelioid cells with a large, clear or slightly granular cytoplasm and centrally located nuclei arranged in nested or trabecular pattern (Figure 2)[13]. These cells are usually arranged around the vessels, which in cutaneous PEComa are present as a rich network that may range from thin capillaries to hyalinized arterioles[7]. Up to 15% of PEComa present cords of neoplastic cells in a desmoplastic stroma[25]. PEComa's cells can also become vacuolated. There have been descriptions of PEComa with presence of multinucleated giant cells and with some degree of nuclear pleomorphism, which have been named as symplastic PEComa[4]. Although pure spindle cell variants may be found, usually spindle cells are intermingled with the epithelioid cells and usually appear in the deeper areas of the neoplasm. Some PEComa may present with slightly pleomorphic multinucleated giant cells with few or no mitotic figures. The more characteristic feature of perivascular epithelioid cells in PEComa is their immunophenotype, which exhibits both smooth muscle cell and melanocytic markers. PEComa express melanocytic markers such as: (1) HBM45 [human melanoma black 45, the most sensitive (expressed in 100% of reported PEComa[8])]; (2) Melan A (72%); and (3) MiTF in most cases. They also express smooth muscle markers such as desmin (typically in a greater degree in cutaneous PEComa when compared with their visceral counterparts[24]); and smooth muscle actin (SMA), that may be the most sensitive marker within this group[4,26-28]. It is important to underline that up to 30% of visceral PEComa stain positive with S100 protein[4].

Pusiol *et al*[29] have recently published a case of a HMB-45 negative tumor that they have named PEComa. In our opinion: (1) microphotographs accompanying this paper are of insufficient quality; and (2) the authors only describe positivity for CD68 and NKI-C3 in neoplastic cells, with no information about immunohistochemical results for muscular markers, such as SMA and desmin; therefore, the diagnosis of PEComa for this case is doubtful[29].

PEComa are characteristically negative for epithelial markers despite their morphologic epithelioid features. Both types of cells, epithelioid and fusiform ones, may express CD1a and cyclin D1[30]. Ultrastructural studies showed that PEComa's cells contain a large cytoplasm with microfilament bundles showing electron-dense con-

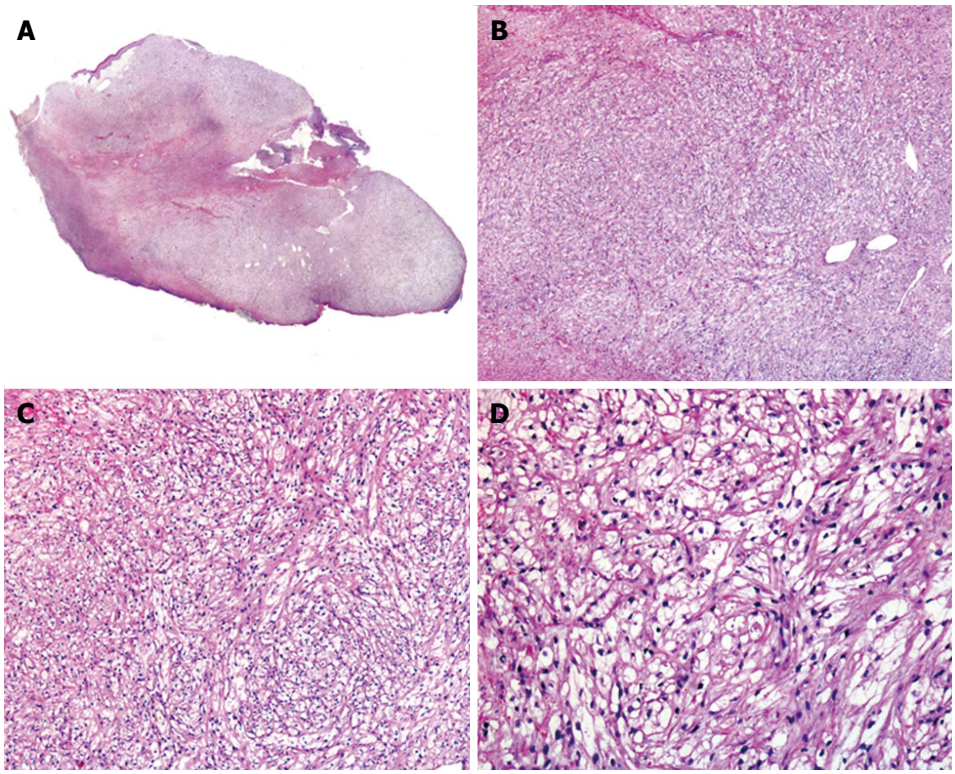

Figure 2 Histopathology of cutaneous perivascular epithelioid cell tumor. A: Low power image of cutaneous perivascular epithelioid cell tumor; B and C: Medium power image showing the lobular arrangement of a neoplasm composed by clear cells; D: Detail of the cells with an ovoid homogeneous nuclei and a clear cytoplasm.

Table 1 Malignant perivascular epithelioid cell tumor's criteria[4]

Features	Definition
Tumor size greater than 5 cm	
Infiltrative growth pattern	Benign (none criteria)
High nuclear grade	Malignant (2 or more features)
Necrosis	Uncertain malignant potential (1)
Mitotic activity > 1/50 high power field	
Aggressive clinical behavior	

densations, numerous mitochondria and membrane-bound dense granules that match premelanosomas[12,26,27].

PEComa's duality lets cells modulate their morphology and immunophenotype. Cases composed mainly of spindled cells usually show a strong expression of actin, but only focal expression of HMB45, whereas cases composed of clear cells usually show strong expression of HMB45 and actin is negative or only focally positive.

Finally, only a few malignant cutaneous PEComa have been reported[7,31]; one of them a scalp lesion that lately metastasized to a regional lymph node[7].

Criteria for diagnosis of "malignant PEComa" have been proposed by Folpe *et al*[4] (Table 1).

CHROMOSOMAL ABNORMALITIES

Recently, recurrent chromosomal alterations have been demonstrated in visceral PEComa. They are related to the genetic alterations of "tuberous sclerosis complex" [due to losses of TSC1 (9q34), TSC2 (16p13.3)], which seem to have a role in the regulation of the Rheb/mTOR/p70S6K pathway[12]. TSC1 is a tumor suppressor gene encoding for hamartin, which creates a complex with TSC2 protein (tuberin) thus with an important role in the mTORC1 pathway.

In the skin, chromosomal losses may be found[5], as well as alterations on chromosome 16p (TSC2); this has been previously reported in angiomyolipomas[5] and also in visceral PEComa, but to date has not been found in the cutaneous lesions, thus lacking evidence of a link between cutaneous PEComa and tuberous sclerosis complex[32]. In visceral PEComa these alterations produce a constitutive activation of the mTORC1 pathway[33]. Some soft tissue PEComa in patients without tuberous sclerosis complex are immunohistochemically positive for TFE3[34,35], but these findings have not yet been detected in cutaneous PEComa, a feature that suggests that the histogenesis of cutaneous PEComa might be different from the visceral ones[36].

Finally, a recent study of Charli-Joseph *et al*[23] using array-based comparative genomic hybridization and a complete immunohistochemical study in 8 cases of primary cutaneous PEComa did not find any chromosomal imbalances or initiating mutations. After their ample immunohistochemical study they have proposed a panel including MITF, NKIC3, SMA, desmin, bcl-1, cathepsin K and 4EB-protein 1 (4EBP1) as the ideal immunohistochemical panel for the evaluation of these

neoplasms[23]. The most interesting immunohistochemical marker within this panel is 4EBP1, as it is a downstream target in the mTOR pathway[37], suggesting, when positive, an activation of the pathway independently of the mutational status of TSC1/TSC2[23].

DIFFERENTIAL DIAGNOSIS

Clear cell myomelanocytic tumor is now included within the PEComa group[9,38], as the previously described as clear cell dermatofibroma[39] although it was considered a different neoplasm for a while[10,40].

Cutaneous PEComa should be differentiated from xanthomatous lesions, granular cell tumors, myoepithelioma, cutaneous meningioma, epithelioid sarcoma, melanocytic neoplasms with balloon cell change, clear cell sarcoma, metastatic clear cell carcinomas (particularly renal cell carcinoma), dermal clear cell tumor and from gastrointestinal stromal tumor.

Xanthomas may be a manifestation of hyperlipidemia; they are histopathologically characterized by a dermal collection of foamy histiocytes and thus they are positive for CD68, CD163 and, in some cases, for adipophilin[41].

Granular cell tumors cells are characterized by a prominent cytoplasm replete with eosinophilic, PAS positive, diastase-resistant granules immunohistochemically characterized for the expression of S-100 protein, PGP9.5, NKIC3, CD68, nerve growth factor receptor 75 and SOX10, which differs from the immunophenotype usually found in cutaneous PEComa; although both neoplasms share MITF-1 positivity the rare congenital granular cell tumors show also richly vascularized stroma[42,43]. In any case, to make the diagnosis even trickier, granular cell tumors may present clear-cell areas, usually as a focal finding, but sometimes occupying most of the tumor[44].

Myoepitheliomas are composed of polygonal shaped cells positive for EMA, calponin, AE1/AE3, SMA and desmin, and S100 protein; but negative for HMB-45, melan-A, tyrosinase and MITF[45].

Primary extracraneal meningioma often presents islands of clear cells and the distinction from cutaneous PEComa is usually straightforward, but as this tumor is typically EMA positive, with a variable positivity for S-100 protein and HMB45 negative, immunohistochemistry may be a useful tool in doubtful cases[46,47].

Epithelioid sarcoma is a malignant neoplasm characterized by polygonal cells with an eosinophilic cytoplasm positive for high and low weight cytokeratins, EMA and vimentin; and negative for S-100 and HMB45[48]. Characteristically, the nuclei of neoplastic cells of epithelioid sarcoma show loss of expression on INI-1.

Melanocytic neoplasms with balloon cells usually present junctional nests and express S100 protein along with other melanocytic markers. Balloon cells are usually a focal finding, although some tumors may appear entirely composed of them[49]. Even when SMA may be positive in desmoplastic melanoma[50,51], the absence of S-100 protein staining and the positivity for SMA favor the diagnosis of PEComa. Recently, a case of pigmented PEComa with presence of focal melanin pigmentation and strong positivity for HMB-45 has been published and may represent a mimicker of melanoma[52].

Neoplastic cells of clear cell sarcoma often show an eosinophilic (rather than clear) cytoplasm and, in challenging cases, the detection of *t*(12;22)(q13;q12), with the resultant EWSR1-ATF1 fusion product, is diagnostic. Some peculiar cases of clear cell sarcoma-like tumor of the gastrointestinal tract presents EWSR1-CREB1 instead of the more commonly found EWSR1-ATF1, thus fluorescence in situ hybridization for EWSR1 gene rearrangement may be also useful[33].

Metastatic clear cell carcinomas express cytokeratins and PEComa is negative for them. Clear cell dermal mesenchymal tumor is usually located on the legs of adults, and histopathologically shows dermal sheets of oval to polygonal cells with abundant clear to slightly granular PAS-negative cytoplasm that is also positive for NKIC3, CD68 and vimentin, whereas melanocytic and muscular markers are consistently negative[53]. Some authors consider that this tumor is possibly associated with PEComa, but still remains considerated as a different entity based on the negativity for melanocytic markers[54]. Finally, Tomasini *et al*[55] published a peculiar neoplasm under the name of eruptive dermal clear cell desmoplastic mesenchymal tumor with perivascular myoid differentiation. This neoplasm showed multiple perivascular spindled to oval cells, intermingled with clear and granular cells as well as prominent desmoplasia, and a high degree of capillary vessels with hemangiopericytoma-like features[55]; this tumor was positive for h-caldesmon, SMA, CD13, CD68 and NKIC3[55].

Visceral PEComa do not express CD34 or c-kit, which is in contrast with GIST. Recently a case of cutaneous metastasis from an adrenal PEComa has been reported showing the same characteristics than a primary cutaneous PEComa, thus making necessary clinicopathologic correlation for a correct diagnosis as the patient presented with widespread metastatic disease[56].

TREATMENT

As most PEComa are benign tumors, surgical removal is curative[1].

A recent review on PEComa located on head and neck suggests that they may be more aggressive, as one of the two malignant cutaneous PEComa and one soft tissue malignant PEComa[57] were in this location.

Besides surgery, drugs inhibiting the activation of mTOR, such as rapamycin, may be useful[58-62]. As patients with tuberous sclerosis have abnormalities in the *TSC2* gene and that activates mTOR leading tumorogenesis, this explain why treatment with rapamycin seems to be useful in the treatment of renal angiomyolipomas and skin lesions of this syndrome, and may be also useful in a subset of PEComa with mTOR activation.

Symplastic PEComas portend an unknown biological

behaviour[63].

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Standardization in laboratory medicine: Adoption of common reference intervals to the Croatian population

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Abstract

Considering the fact that the results of laboratory tests provide useful information about the state of health of patients, determination of reference value is considered an intrinsic part in the development of laboratory medicine. There are still huge differences in the analytical methods used as well as in the associated reference intervals which could consequently significantly affect the proper assessment of patient health. In a constant effort to increase the quality of patients' care, there are numerous international initiatives for standardization and/or harmonization of laboratory diagnostics in order to achieve maximum comparability of laboratory test results and improve patient safety. Through the standardization and harmonization processes of analytical methods the ability to create unique reference intervals is achieved. Such reference intervals could be applied globally in all laboratories using methods traceable to the same reference measuring system and analysing the biological samples from the populations with similar socio-demographic and ethnic characteristics. In this review we outlined the results of the harmonization processes in Croatia in the field of population based reference intervals for clinically relevant blood and serum constituents which are in accordance with ongoing activity for worldwide standardization and harmonization based on traceability in laboratory medicine.

Key words: Calibration traceability; Applicability of reference intervals; Harmonization and standardisation; External quality assessment; International Federation of Clinical Chemistry enzyme reference methods; Aminotransferase "common" reference intervals; Creatinine enzymatic method

Core tip: The main goal of medical laboratories is to be able to support the clinicians with the best achievable quality in all laboratory results and reports. Ongoing evaluation and improvement processes are essential to ensure performance in compliance with the highest

professional and accreditation standards in order to provide optimal health benefit for the patient. In this review we outlined the results of the harmonization processes in Croatia in the field of population based reference intervals for clinically relevant biochemical constituents which are in accordance with an ongoing activity for worldwide standardization and harmonization based on traceability in laboratory medicine.

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INTRODUCTION

The population based reference intervals for healthy subjects are of outmost importance for the transversal clinical interpretation of laboratory test values[1-4]. In spite of ongoing improvement processes there are still huge differences in the analytical methods used, as well as in the associated reference intervals which could consequently significantly affect the proper assessment of patient health[5,6]. The International Organization for Standardization (ISO) standard 15189 require that "biological reference intervals shall be periodically reviewed"[7], while according to the directive of the European Union on *in vitro Diagnostics Medical Devices* the manufacturers have to provide detailed information on reference intervals[8].

Considering the fact that the results of laboratory tests provide useful information about physiological changes and the state of health of patients, determination of reference value is considered an intrinsic part in the development of laboratory medicine.

PRODUCTION OF POPULATION BASED REFERENCE INTERVALS IN CROATIA

Availability of the right interpretation of laboratory test results and reports is essential to ensure the optimum patient outcome. For this reason investigations of biological variation and the appropriate reference intervals for different clinically relevant biochemical constituents have been the subject of investigation of many laboratories, mostly throughout Western Europe and North America, over the last few decades. For the first time in our country the health associated reference intervals were produced for 34 blood and serum constituents according to the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) recommendations proposed by the Clinical Laboratory Standard Institute (CLSI) C28 document[9] on the representative reference sample group of 2246 adults and 998 school children and adolescents, age 8-70 years, from the territory of Zagreb and its surrounding, in the period from 1998 to 2000[10-15].

Reference intervals for glucose, potassium, sodium, chloride, magnesium, iron, zinc, total proteins and electrophoretic fractions of total serum proteins, enzyme activities, total and low density cholesterol and triacylglycerols as well as for hematological and coagulation parameters were obtained by using nonparametric methods to estimate 2.5 and 97.5 percentiles of age and sex distribution as upper and lower normal reference intervals. Owing to related achievements, the Department of Medical Biochemistry and Laboratory Medicine at the University Hospital "Merkur" Zagreb, was designated by the Ministry of Health of the Republic of Croatia as the national Reference Center for the Production of Reference Values in the Field of General Medical Biochemistry in 2000. Today's activities of the Reference Centre are directed to harmonization of laboratory test results based on metrological criteria and traceability concept in laboratory medicine and validation of the applicability of the global "common" reference intervals to the Croatian population.

HARMONIZATION OF LABORATORY TEST RESULTS IN CROATIA

In order to contribute to the harmonization process of laboratory test results at the national level, in 2004 the Croatian Society of Medical Biochemists [Committee for External Quality Assessment (EQA) of MBLs], Croatian Chamber of Medical Biochemists and Department of Clinical Chemistry and Laboratory Medicine University Hospital Merkur-Reference centre of the Ministry of Health for the production of reference values in the field of general medical biochemistry have started the project of harmonization of laboratory test results for the following parameters: Metabolites and substrates, enzymes, electrolytes, microelements, proteins, routine urine analysis, complete blood count with differential and laboratory coagulation.

Project consisted of two main phases. The main goal of the first phase was to achieve analytical comparability of the tests results and reports between the medical biochemistry laboratories of the primary, secondary and tertiary level of medical care in Croatia based on implementation of recommended analytical methods. Second phase key goal was to achieve the highest possible level of clinical comparability of the test results by the application of the health associated population based reference intervals produced on representative reference sample group of Zagreb and its surroundings to all Croatian medical biochemistry laboratories.

The long-term evaluation of national EQA results in the period from 1998 to 2003 has shown that medical biochemistry laboratories use different analytical methods for the same analytes and that some of them were not able to fulfil the required analytical quality specification. For this reason, in the first phase of the project the

application of the same analytical methods for the routine blood and serum constituents in all medical biochemistry laboratories in Croatia was recommended. After applying recommended analytical methods and complying with established criteria for analytical quality of the results through the national program of EQA, conditions have been met for the second phase of the project - clinical comparability of obtained results. In 2005 the Croatian Chamber of Medical Biochemists recommended the use of health associated population based reference intervals produced on representative reference sample group of Zagreb and its surroundings to all Croatian medical biochemistry laboratories using the same analytical methods with acceptable performance evaluated through national EQA programme[16,17]. For the paediatric population, unique reference intervals were recommended according to the literature data.

THE ROLE OF LONG TERM EVALUATION OF NATIONAL EQA IN HARMONIZATION OF REFERENCE INTERVALS IN CROATIA

Proficiency testing programs in Croatia have been continuously performed since 1973, by the Committee for EQA, which in 2012 outgrow in the CROQALM - Croatian centre for quality assessment in laboratory medicine conducted by the Croatian Society for Medical Biochemistry and Laboratory Medicine, a non-profit, non-governmental organization dedicated to operate a nationwide quality assessment in laboratory medicine according to the international standard for the providers of interlaboratory comparisons, ISO/IEC 17043:2010 - Conformity assessment - General requirement for proficiency testing, which was adopted as Croatian norm in 2010. Croatian Society for Medical Biochemistry and Laboratory Medicine as an independent organizer of the EQA of medical biochemistry laboratories, became a full member of European Organization For External Quality Assurance Providers in Laboratory Medicine (EQALM) in 1998. Many of Croatian medical biochemistry laboratories actively participate in international measurement evaluation projects in the field of medical biochemistry and post-analytical automated haematology under the auspices of EQALM in order to achieve a high degree of interlaboratory comparability and improve the analytical performance of laboratory tests required for patient care.

The national external quality asessment programme for medical biochemistry laboratories is formed modulary including laboratory tests in the field of medical biochemistry, laboratory haematology and coagulation, urinalysis, acid base status and ionized electrolytes, thyroid hormones, tumour markers and glycated haemoglobin and is organised three times per year. For acceptability of laboratory test results an internationally accepted hierarchical approach to analytical quality goals based on metrological principles, biological variation and diagnostic needs is used. Certificate for the participation in the national EQA is issued annually to each laboratory.

The long-term evaluation of the obtained results presented in the publications in the relevant professional and scientific periodicals, workshops organised at the national congresses, symposia and meetings as well as presentations at the international meetings[18-21] show that national proficiency testing programs have an important role in improving analytical quality and working conditions in the medical biochemistry laboratories in Croatia and became the basis of overall activities in the field of harmonisation of laboratory test results and transmission of the international recommendations into the national expert practice. The significant contribution to the interlaboratory comparability of the results comes also through the legislative regulations by Law on Medical - Biochemical Activities in 2003, according to which the participation of medical biochemistry laboratories in the national EQA programmes became mandatory.

Medical biochemistry laboratories in Croatia also participate in the different EQA schemes in the field of clinical chemistry, laboratory haematology and coagulation organized by international EQA providers: Labquality (World Health Organization Collaborating Centre for Education and Training in Laboratory Quality Assurance) Helsinki, Finland; United Kingdom National EQA Scheme for Haematology and Blood Coagulation; Sheffield, United Kingdom; Reference Institute for Bioanalytics Bonn, Germany; ECAT Foundation (External Quality control of diagnostic assays and tests with a focus on Thrombosis and Haemostasis) Amsterdam, The Netherlands; INSTAND e.V. (Society for Promoting Quality Assurance in Medical Laboratories e.V.), Düsseldorf, Germany in order to provide performance in compliance with the highest professional standards, reduce laboratory errors and improve patient safety as the most important priority in laboratory medicine.

GLOBAL STANDARDIZATION/ HARMONIZATION IN LABORATORY MEDICINE

In a constant effort to increase the quality of patients' care, laboratory diagnostics are of great importance. In this regard there are numerous international initiatives for standardization and/or harmonization of laboratory diagnostics in order to achieve maximum comparability of laboratory test results, because non-standardized and/or non-harmonized results can lead to diagnostic errors and thereby reduce patient safety[22]. Consequently, the main impetus for standardization and/or harmonization in laboratory medicine is to increase patient safety, but other reasons include the regulatory requirements such as accreditation in laboratory medicine, as well as the benefits of information technology including the possibility of creating an electronic patient record[22]. To achieve this result it is necessary to harmonize the entire

Table 1 Reference intervals for creatinine concentrations[31]

Age (gender) group	Percentile value, μmol/L	
	2.5th	97.5th
Common reference intervals for global application		
Cord sera	46	86
Term neonates 0-14 d	27	81
2 mo - < 1 yr	14	34
1 yr - < 3 yr	15	31
3 yr - < 5 yr	23	37
5 yr - < 7 yr	25	42
7 yr - < 9 yr	30	48
9 yr - < 11 yr	28	57
11 yr - < 13 yr	37	63
13 yr - < 15 yr	40	72
Adult (males)	64	104
Adult (females)	49	90
Reference intervals in the reference sample group of Croatian population (n = 240)		
Adult (males)	54	107
Adult (females)	50	93

laboratory examination including analytical processes which are under the direct control of laboratory professionals as well as processes that are outside of such control such as request appropriateness as a part of pre-preanalytical processes and the correct use and interpretation of the obtained laboratory test reports as a most important part of post-postanalitical processes.

As part of the process of standardization of analytical methods and the establishment of reference measurement systems in laboratory medicine, in 2002 the Joint Committee for Traceability in Laboratory Medicine (JCTLM) was established in order to coordinates the activities of the International Bureau of Weights and Measures (Bureau International des Poids et Mesures), the IFCC, the International Laboratory Accreditation Cooperation, the organizers of EQA and the manufacturers of equipment and reagents [*in vitro* diagnostics (IVD)][22,23]. As a result of all these activities the JCTLM has created a database of accepted and available reference materials, reference analytical methods and accredited reference laboratories. In addition, despite the opinion of a part of the laboratory experts that the concept of measurement traceability could not be introduced in the area of laboratory medicine except in rare cases[24], it was shown that the application of metrological traceability has a great practical potential and global value. This was confirmed through[24]: The establishment of a reference measurement system (JCTLM); Development of analytical methods and related reagents (IVD) in accordance with traceability chain; Producing traceable, multicenter reference intervals; Introducing the comutabile control samples in EQA schemes in order to objectively assess the level of achieved analytical accuracy; Defining target values for analytical methods used; Rejection of the application of non-specific methods of insufficient quality.

Through the standardization of analytical methods the ability to create unique reference intervals is achieved. Such reference intervals could be applied globally in all laboratories using methods traceable to the same reference measuring system and analysing the biological samples from populations with similar socio-demographic and ethnic characteristics[23,25].

For the complex analytes for which the laboratory test results often are not expressed in SI-, but in arbitrary units the concept of harmonization has been proposed based on the "Step-Up" design[26,27]. This essentially comprises a sequence of method comparisons with selected sets of commutable samples. The outcome of each phase informs the decision as to whether the step-up to the next phase should be undertaken. The biggest disadvantage of this process is the limited amount of commutabile clinical samples required to maintain the process of harmonization[26,27]. In 2010, in order to launch international initiatives for the harmonization process in the laboratory medicine the International Consortium for Harmonization of Clinical Laboratory Results, (www.harmonization.net) based in the American Association for Clinical Chemistry was founded. This ensures a global infrastructure with the aim of defining a systematic approach to determining the list of the complex analytes for which there are no higher-order reference measurement procedures and for which it was unlikely that such procedures could be developed[27] in order to increase patient safety through the best achievable quality and comparability of all laboratory test results.

APPLICABILITY OF COMMON REFERENCE INTERVALS FOR SERUM CREATININE CONCENTRATIONS TO THE CROATIAN POPULATION

In order to harmonize the serum creatinine results and their interpretation the applicability of recommended "common" reference intervals for creatinine concentrations was evaluated[28-30]. Serum creatinine concentrations were measured using specific enzymatic method traceable to the IDMS method in comparison to the uncompensated Jaffe kinetic creatinine method[31]. The representative reference sample group consisted of 240 healthy subjects who were "a priori" selected in accordance with the IFCC recommendation. The obtained results were almost identical as the recently recommended "common" reference intervals for global application by the IFCC Committee on Reference Intervals and Decision Limits[32].

Based on the obtained results (Table 1) it is recommended that the "common" reference intervals could be used for creatinine measurement in all Croatian medical-biochemistry laboratories employing standardized, specific enzymatic method. The most important prerequisite is that their analytical performance meet the recommended performance goal of < 10% total error[33-35]. According to the CLSI approved guideline, validation of reference intervals is advisable[36].

The introduction of common reference intervals produced using specific enzymatic method should cause the disappearance of different intervals for creatinine results depending on the analytical method used which is in accordance with the National Kidney Disease Education Program recommended that estimated glomerular filtration rate has to be routinely reported along with specific serum creatinine measurements[37-39].

TRANSFERABILITY OF ASPARTATE AND ALANINE AMINOTRANSFERASE COMMON REFERENCE INTERVALS TO THE CROATIAN ADULT AND PEDIATRIC POPULATION

According to the standardization of enzyme catalytic activity concentration measurements using IFCC reference methods and production of standardized reference intervals, the evaluation of the transferability of IFCC recommended "common" reference intervals for aspartate and alanine aminotransferase to the Croatian population was performed by the Department of Clinical Chemistry and Laboratory Medicine University Hospital Merkur-Reference centre of the Ministry of Health for the production of reference values in the field of general medical biochemistry, Zagreb, Croatia.

The reference group consisted of 120 healthy subjects (40 adults and 60 paediatric samples, between 1-19 years of age) selecting a posterior according to the strictly defined criteria. In standardised pre-analytical conditions the catalytic activity concentration for serum aspartate and alanine aminotransferase were measured using IFCC reference methods on the Beckman Coulter AU 680 biochemical analyser.

Analytical methods used in this study are accredited according to ISO 15189 and the results were confirmed through participation of the Department of Medical Biochemistry and Laboratory Medicine Merkur University Hospital in the International EQA schemes organized by Labquality WHO Collaborating Centre for Education and Training in Laboratory Quality Assurance, Helsinki, Finland. The reference intervals were validated as recommended by the CLSI[36].

The obtained results showed that in age groups which represent the local adult healthy population, 18 to 20 subjects (95%-100%) were within the recommended IFCC common reference intervals for aspartate as well as for alanine aminotransferase catalytic activity concentration. The obtained results for paediatric samples showed that 18 to 20 subjects (95%-100%) where within the evaluated reference intervals for the group between 10 to 12 years of age. In the age group between 13-19 years 55% to 65% of results were within the evaluated reference intervals while the other results were below the reference intervals. Verification of reference intervals for aminotransferases for Croatian adult and pediatric patients using IFCC recommended analytical methods in comparison to previously produced reference intervals recommended by Croatian Chamber of Medical Biochemists in 2007, are presented in Tables 2 and 3 together with related references[10,14,15,40-44].

The obtained results confirmed that the IFCC recommended common reference intervals for aspartate and alanine aminotransferase activity concentrations are appropriate for the adult Croatian population. The verification of reference intervals for the paediatric population obtained with IFCC recommended reference methods have to be confirmed with multiple local validations in order to become widely used[45].

In 2014, as a part of the ongoing harmonisation project the Croatian Chamber of Medical Biochemists has recommended specific enzymatic method to be used as routine analytical method for the measurement of serum creatinine concentrations and IFCC recommended methods for the measurement of aspartate aminotransferase and alanine aminotransferase activity concentrations. Based on the evaluation of the reference intervals and verification studies application of "common" reference intervals to the Croatian population was recommended. The results of these long-term evaluation and improvement processes as well as interlaboratory variability of the obtained results are clearly demonstrated through national EQA program which is obligatory for all medical biochemistry laboratories and is one of quality indicators in scope of the external professional audit of medical biochemistry laboratories in Croatia.

CONCLUSION

Since the process of standardization and/or harmonization give a very important contribution to raising the overall quality of laboratory diagnostics and thus significantly improves the level of health care of patients, it is necessary to constantly encourage this process through the active involvement of manufacturers, regulatory authorities, the organizers of EQA and medical and laboratory experts.

The highest priority in laboratory work in this process is to constantly raise the level of patient safety, thus reducing the risk of possible laboratory error that can adversely affect the process of treatment or be the cause of possible fatal outcome[46]. In this respect the introduction of quality management system according to international standard ISO 15189 in clinical laboratories gives a strong contribution to the timely elimination of all potential errors that could compromise the safety of patients and supports preventive measures and activities to resolve possible errors and to ensure the desired outcomes of treatment. The International Standard ISO 15189 has been accepted as Croatian norm in 2003. Accreditation of medical laboratories is carried out on voluntary basis by the Croatian Accreditation Agency (HAA) which has a full membership in European co-operation for Accreditation. Up to now HAA has accredited 8 medical biochemistry laboratories in the

Table 2 Verification of reference intervals for alanine amino-transferase for Croatian patients

Analyte	Method	Unit	Reference interval			Ref.	Verification (%)
			Sex	Age (yr)	Interval		
ALT	Photometry UV, IFCC method, 37 ℃, TRIS buffer, L-alanine, α-ketoglutarate, pyridoxal phosphate, NADH, lactate dehydrogenase, pH 7.15: CCMB recommendation for the Croatian population	U/L	Male, female	0-2	11-46	[40]	
			Male, female	3-7	9-20	[40]	
			Male, female	8-12	11-37	[10,15]	
			Male	13-19	10-33	[10,15]	
			Female	13-19	10-29	[10,15]	
			Male	⩾ 20	12-48	[14]	
			Female	⩾ 20	10-36	[14]	
	IFCC reference measurement procedure (IFCC RMS), IFCC IRMM reference material ERM-AD454 (2002): Common multicentric reference intervals for the adult population, 2010	U/L	Male	18-85	9-59	[41]	96
			Female	18-85	8-41		96
	Pediatric reference intervals						
	CALIPER, Canada, 2012; IFCC reference measurement procedure, Abbott Architect		Male, female	0 < 1	5-51	[42]	
			Male, female	1 < 13	11-30		90
			Male	13 < 19	10-33		55
			Female	13 < 19	8-24		65
	Denmark (NORIP), 2012; IFCC reference measurement procedure, Roche Modular		Male	5-8	8-27	[43]	
			Male	9-13	8-37		
			Male	14-18	8-47		
			Female	5-18	8-32		
	Germany (a posteriori, ambulance and hospital populations) 2009; IFCC reference measurement procedure, Roche Modular		Male, female	0-1	< 49	[44]	
			Male, female	1-3	< 29		
			Male, female	4-6	< 39		
			Male, female	7-12	< 44		
			Male	13-17	< 51		
			Female	13-17	< 45		

ALT: Alanine amino-transferase; IFCC: International Federation of Clinical Chemistry and Laboratory Medicine; CCMB: Croatian Chamber of Medical Biochemists; IRMM: Institute for reference materials and measurements; RMS: Reference measurement system; CALIPER: Canadian laboratory initiative on pediatric reference intervals.

Table 3 Verification of reference intervals for aspartate amino-transferase for Croatian patients

Analyte	Method	Unit	Reference interval			Ref.	Verification (%)
			Sex	Age (yr)	Interval		
AST	Photometry UV, IFCC method, 37 ℃, TRIS buffer, L-aspartate, α-ketoglutarate, pyridoxal phosphate, NADH, malate dehydrogenase, lactate dehydrogenase, pH 7.65: CCMB recommendation for the Croatian population	U/L	Male, female	0-2	26-75	[40]	
			Male, female	3-7	24-49	[40]	
			Male, female	8-12	14-39	[10,15]	
			Male	13-19	11-38	[10,15]	
			Female	13-19	14-32	[10,15]	
			Male	⩾ 20	11-38	[14]	
			Female	⩾ 20	8-30	[14]	
	IFCC reference measurement procedure (IFCC RMS), IFCC IRMM reference material ERM-AD457 (2002): Common multicentric reference intervals for the adult population, 2010	U/L	Male, female	18-85	11-34	[41]	94
	Pediatric reference intervals						
	CALIPER, Canada, 2012; IFCC reference measurement procedure, Abbott Architect		Male, female	1 < 7	26-55	[42]	100
			Male, female	7 < 12	22-41		96
			Male	12 < 19	18-40		96
			Female	12 < 19	17-33		96
	Denmark (NORIP), 2012; IFCC reference measurement procedure, Roche Modular		Male, female	5-18	17-46	[43]	
	Germany (a posteriori, ambulance and hospital populations) 2009; IFCC reference measurement procedure, Roche Modular		Male, female	0-1	< 77	[44]	
			Male, female	1-3	< 71		
			Male, female	4-6	< 53		
			Male, female	7-12	< 48		
			Male	13-17	< 42		
			Female	13-17	< 44		

AST: Aspartate amino-transferase; IFCC: International Federation of Clinical Chemistry and Laboratory Medicine; CCMB: Croatian Chamber of Medical Biochemists; IRMM: Institute for reference materials and measurements; RMS: Reference measurement system; CALIPER: Canadian laboratory initiative on pediatric reference intervals.

Republic of Croatia.

The introduction of standardized analytical methods reduces interlaboratory differences and requires the production of standardized, traceable reference intervals through multicenter studies with a large number of reference individuals, which should reflect only possible ethnic differences between the examined populations. The implementation of such population based reference intervals to the local population based on verification studies will significantly improve the quality of interpretation of laboratory results on a global level resulting in optimal health benefits for the patient.

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Study design in evidence-based surgery: What is the role of case-control studies?

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Abstract

Randomized controlled trials (RCTs) are the gold standard in terms of study design, however, in the surgical setting conducting RCTs can often be unethical or logistically impossible. Case-control studies should become the major study design used in surgical research when RCTs are unable to be conducted and definitely replacing case series which offer little insight into surgical outcomes and disease processes.

Key words: Research studies; Case-control studies; Randomized clinical trials; Bias; Sample size

Core tip: Case-control studies should be utilized more often in the surgical setting for research purposes. They offer many advantages to other study designs, especially when the option of conducting a randomized clinical trial may be impractical or not ethically feasible.

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INTRODUCTION

The hierarchy of study design is well ingrained in determining the quality and subsequent acceptance of clinical evidence (Figure 1). Randomised controlled trials (RCT) are considered the gold standard study design and the "most scientifically rigorous method for hypothesis testing", with results from many non-randomised trials prejudiced by doubts of study reliability, bias and accuracy[1-3]. Yet in certain aspects of surgery, RCTs may be difficult to conduct and indeed the number of surgical RCTs is known to be limited in comparison[4].

RCTs involve the comparison of outcomes after random allocation of a particular intervention to a patient group with a control group whilst case-control studies (CCS) involve observing outcome differences between

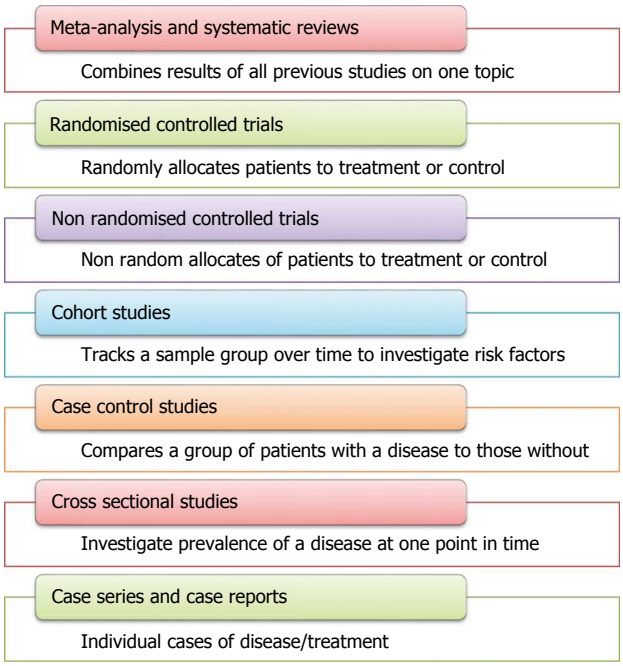

Figure 1 Hierarchy of study design.

patients with a particular disease (cases) and those without the disease (control). It is commonly accepted that results from RCTs provide superior evidence in the evaluation of a therapeutic intervention when compared to CCS. However, there are many considerations that result in flaws in this concept especially in surgery. Difficulties in standardising surgical technique, variable learning curves in introducing new or modifications of an operation and difficulties in recruiting patients leading to underpowered studies need to be recognised[5]. In fact results from poorly designed RCTs can have the undue advantage of being perceived and accepted as the "superior study design" with more robust findings[6]. The aim of this paper is to explore various factors influencing the role of CCS in the surgical context and provide recommendations to improving the quality of CCS.

POWER

The strength of CCS lie in its ability to recruit larger sample sizes, resultant increase in the power of studies, lower cost and the ability to be conducted in "greater timeliness" (Table 1)[7]. CCS also have the ability to report rare infrequent adverse effects, *e.g.*, bile duct injuries in laparoscopic cholecystectomies[8,9]. As CCS may be performed by researchers with limited resources, larger patient populations are able to be recruited compared with RCTs which generally require more expert support from epidemiologists and require financial support[10]. Lack of funding and resource constraints have been cited as major obstacles in conducting RCTs[11]. Inadequate sample sizes lead to underpowered RCTs which may miss clinically important benefits and lead to type Ⅱ error[12]. Type Ⅱ error is the failure to reject the null hypothesis when it is false, *i.e.*, False negative results[12].

Table 1 Advantages and disadvantages of case control studies

Advantages of case control studies	Disadvantages of case control studies
Ability to investigate low incidence outcomes	Risk of bias
Ability to recruit large sample size	Confounding factors
Relative ease and efficiency	Requires careful selection of controls
May be conducted in shorter time frame	Weaker evidence of causality (20)
Relatively low cost	Blinding is not possible

CLINICAL APPLICABILITY

A particular strength of CCS is the inclusion of data from practical clinical scenarios. RCTs, whilst limiting potential confounding variables, provide evidence from data collected from highly rigid experimental models[13]. In investigating certain surgical techniques such as laparoscopic cholecystectomy, strict criteria such as those excluding obese patients and patients with multiple comorbidities are likely to lead to results inconsistent with the clinical setting and limit the practicality of findings. In patients who have rare or life threatening illnesses, it will be difficult to include them in RCT[6]. In addition unlike CCS, RCT also tend to limit the spectrum of disease represented compared to observational studies[2,6].

RANDOMISATION AND CONFOUNDERS

Non-randomised observational studies such as CCS and cohort studies are more prone to bias than RCT due to lack of randomisation. The randomisation process aims to minimise systematic error and eliminate or at least equilibrate confounding factors between both treatment and control groups. It is more difficult for observational studies to allow for this equilibration and hence is more prone to bias. Without randomisation, it may be unclear why certain patients were assigned to a particular intervention whilst others were not[9]. However, whilst randomisation can limit bias, it may not be feasible or ethical in the surgical context. For example, it may be unethical to deny one group of patients the treatment benefits of well established "gold standard" interventions[14]. In addition, it may be difficult to recruit patients who will leave their choice of treatment up to chance alone and accept the process of randomisation[11].

Whilst it is more difficult for CCS to account for confounding factors, it is not impossible without randomisation. Matching controls with cases is one potential method[15]. Matching where controls are specifically selected for their similarity to the treatment group in particular characteristics such as age, sex, socioeconomic status, body mass index, *etc.*, can be used to equilibrate potential confounders in CCS.

Allocation concealment and blinding

Furthermore in surgery, allocation concealment and blinding may be impractical and unethical. In most major

surgical procedures, it would be unethical to expose patients in the control groups to the risks of sham operations. Whilst various techniques have been used in the blinding of patients in surgery including the use of multiple wound dressings over intact skin, the efficacy of such blinding techniques is unclear.

Bias

The concern that observational studies can bias evidence by finding stronger treatment associations than RCTs has been reported in the literature[7,16]. However comparisons between results for observational and RCTs in other studies have shown results to be similar between the two in most outcomes[7]. For example one study analysed the results of meta-analyses comparing RCTs and well-designed observational studies (cohort and case control studies) on a range of treatments including hypertension treatment and CHD, Bacillus Calmette-Guerin vaccine in tuberculosis, mammography screening for breast cancer and found results from observational studies "did not systematically overestimate the magnitude of exposure-outcome associations reported in RCTs"[2,7]. An explanation for the noted differences in some studies between RCT and CCS potentially results from less robustly designed CCS were used to generate generalised conclusions regarding observational studies[2].

Recommendations to improve CCS

It would be imprudent to argue that CCS provide a superior level of evidence to RCT. However, CCS can often provide additional and more clinically relevant evidence that can complement data derived from RCTs. There are various means of ensuring high quality CCS. Recommendations to ensuring sound CCS evidence include: (1) encourage use of STROBE statement to ensure adequate reporting of outcomes[7]; (2) develop an exhaustive database of baseline characteristics and variables during data collection stage of CCS; (3) design CCS to test the clinical applicability and generalizability of results from RCT rather than formulating hypothesis to investigate[17-19]; (4) ensure adequate statistical power and sample size by performing sample size and power calculations prior to the initiation of studies; (5) appropriate statistical techniques for the clinical question, *e.g.*, Propensity analysis to match patients, use of risk adjusted statistical models; and (6) encourage sound methodology techniques such as intention to treat and adequate follow-up.

CONCLUSION

Well-designed RCTs undoubtedly provide powerful estimates of treatment effects. However, they are time-consuming, costly, difficult to conduct especially in surgery and can be misinterpreted when data is extrapolated outside the experiment sample. CCS on the other hand have the ability to recruit large sample sizes, are more efficient to conduct and allow for the examination of variables in the clinical setting. It is unfortunate that CCS are often undervalued and under-utilised in surgery. RCT and CCS provide evidence that is complementary to each other. Greater understanding is required in appraising RCT and CCS in the surgical environment.

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Role of positron emission tomography-computed tomography in non-small cell lung cancer

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Abstract

Lung cancer is the leading cause of cancer-related mortality worldwide. Non-small cell carcinoma and small cell carcinoma are the main histological subtypes and constitutes around 85% and 15% of all lung cancer respectively. Multimodality treatment plays a key role in the successful management of lung cancer depending upon the histological subtype, stage of disease, and performance status. Imaging modalities play an important role in the diagnosis and accurate staging of the disease, in assessing the response to neoadjuvant therapy, and in the follow-up of the patients. Last decade has witnessed voluminous upsurge in the use of positron emission tomography-computed tomography (PET-CT); role of PET-CT has widened exponentially in the management of lung cancer. The present article reviews the role of 18-fluoro-deoxyglucose PET-CT in the management of non small cell lung cancer with emphasis on staging of the disease and the assessment of response to neoadjuvant therapy based on available literature.

Key words: Positron emission tomography; Diagnostic imaging; Neoplasm staging; Carcinoma; Non-small-cell lung cancer; Lung neoplasms

Core tip: The evidence is evolving for the role of positron emission tomography-computed tomography

(PET-CT) in the management of non-small cell lung cancer (NSCLC). Available literature supports the use of PET-CT in the staging of NSCLC to have better disease staging (assessment of mediastinal and extra-thoracic disease). Detection of abnormal mediastinal nodes at various basins is the potential advantage of PET-CT for better targeted biopsy and it may lead to reduction in futile surgical interventions. The role of PET-CT in the prediction and assessment of response to neoadjuvant therapy needs further studies.

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INTRODUCTION

As per GLOBOCAN 2012 data, lung cancer is the leading cause of cancer related death worldwide; an estimated 1.8 million new lung cancer cases occurred in 2012, accounting for about 13% of total cancer diagnoses[1]. Non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma are the main histological subtypes and constitutes around 85% and 15% of all lung cancer respectively[2]. Multimodality treatment is the key to successful management of lung cancer depending upon the histological subtype, stage of the disease, and performance status of the patient. Imaging modalities play an important role in the diagnosis and accurate staging of the disease, in assessing the response to the neoadjuvant therapy, and in the follow-up of the patients. The role of positron emission tomography-computed tomography (PET-CT) has widened exponentially during the last decade in the management of solid tumors, and lung cancer is no exception to this trend. In the present article, we review the role of 18-fluoro-deoxyglucose (FDG) PET-CT in the management of NSCLC with emphasis on the staging of the disease and the assessment of the response to neoadjuvant therapy.

ROLE OF FDG PET-CT IN THE STAGING OF LUNG CANCER

Accurate staging is essential in formulating an optimal management plan for the patient, predicting the prognosis of the disease, and to evaluate and compare the results of various clinical studies by providing a uniform staging terminology across the centers. Staging of NSCLC incorporates assessment of primary tumor, regional lymph nodes and distant sites. Being a whole-body imaging technique, PET-CT has proved to be an enticing option to assess the loco-regional extent and distant sites in a single non-invasive examination. Moreover, combination of functional and anatomical imaging in a PET-CT examination provides greater accuracy in the disease staging.

Primary tumor

A radiologic imaging is required in the assessment of extent of primary tumor. Contrast enhanced computed tomography (CECT) of the chest is traditionally considered the standard imaging modality for delineation of anatomical extent of the primary tumor (Figure 1). At times, magnetic resonance imaging (MRI) is also needed in case of superior sulcus involvement or mediastinal involvement (assessing the relation to heart or great vessels). Because of poor spatial resolution, PET-CT does not offer much advantage over conventional CT/MRI. However, PET-CT has been shown to be superior to CT/MRI in assessing tumor size when there is associated post-obstructive atelectasis or consolidation[3]. Pawaroo *et al*[4], in their study of 59 patients of NSCLC, showed that PET was better than CT with either soft-tissue or lung windows in delineating primary NSCLC if surrounding collapse or consolidation is present. They cautioned that PET may not be reliable for assessment of alveolar cell carcinoma owing to low FDG accumulation. This is to be highlighted that accurate primary tumor is useful for radiotherapy planning if consolidation or collapse surrounds the primary tumor.

Another potential advantage of PET-CT over the conventional imaging is its ability to diagnose pleural disease. Though presence of malignant pleural disease confers a M1 disease and precludes curative surgery; post-obstructive pneumonia related benign effusion should not be erroneously diagnosed as malignant. Conventional imaging modalities like CT and MRI are able to detect pleural thickening or nodularity; however, they are limited in their capacity to differentiate malignant from benign growths with a reasonable amount of certainty[5]. In an analysis of FDG PET-CT images of 33 lung cancer patients with pleural effusion, Kim *et al*[6] suggested that FDG PET/CT can be used as a reliable and noninvasive method for the differentiation of malignant and benign pleural disease in patients with NSCLC. Similar results were also reported by Gupta *et al*[7], they reported PET-FDG imaging is a highly accurate and reliable noninvasive test to differentiate malignant from benign pleural effusion and/or pleural involvement in patients with lung cancer (sensitivity, specificity, and accuracy of 88.8%, 94.1% and 91.4% respectively). This is also worth mentioning here that thoracocentesis may not prove to be futile in up to 30%-40% cases of malignant pleural effusion[8]. In malignant pleural effusion, ^{18}F-FDG PET was found to have a sensitivity of 88.8%, a specificity of 94.1%, a positive predictive value of 94.1%, a negative predictive value 88.8% and an accuracy of 91.4%[9]. Schaffler *et al*[10], evaluated the accuracy of fluorine ^{18}F-FDG PET-CT in differentiation of pleural malignancy and cancer unrelated pleural disease in patients with NSCLC and other pleural abnormalities; they found that sensitivity, specificity, positive predictive value (PPV), negtive predictive value (NPV), and accuracy of FDG PET was 100%, 71%, 63%, 100%

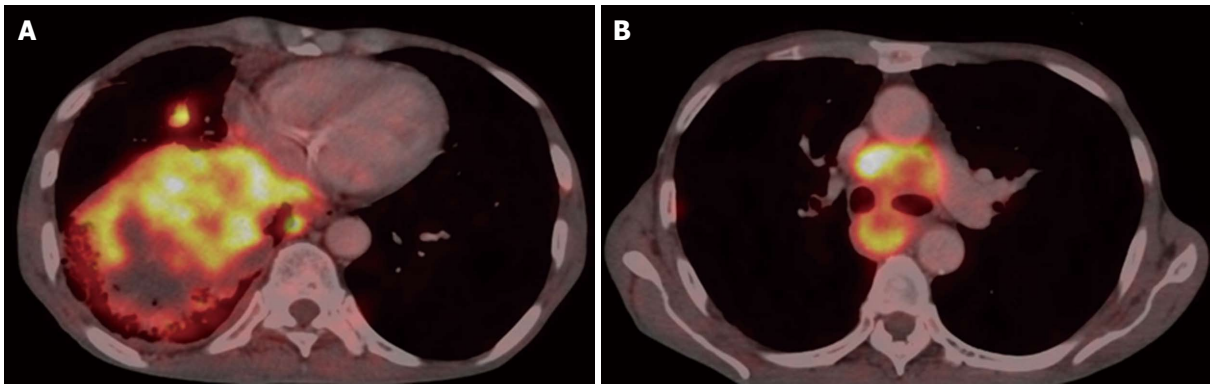

Figure 1 Large soft tissue density mass with heterogeneously increased 18-fluoro-deoxyglucose uptake in the right lung (A) and conglomerated 18-fluoro-deoxyglucose avid paratracheal, subcarinal lymph nodes (B).

and 80%; and those of CT and FDG PET combined, was 100%, 76%, 67%, 100% and 84%. It should, however, be emphasized that all efforts should be made to confirm the metastatic nature of pleural effusion cytologically or by thoracoscopy before committing the patient for a non-curative option.

Regional nodal staging

Undoubtedly, lymph node (N) status is the most important prognostic variable in lung cancer. Accurate mediastinal staging is important to decide optimum management plan for the patient. Presence of mediastinal lymphadenopathy has the potential to change the management approach in NSCLC. CECT determines the nodal staging on the basis of morphological characteristics. Though a number of criteria have been used in various studies to define metastatic node on CT, most widely used criteria is short axis diameter of more than 1 cm on transverse scan[11]. In a review of three studies including 152 patients total, Toloza *et al*[12] concluded that the sensitivity, specificity, positive predictive value and negative predictive value of PET-CT in detecting mediastinal staging ranged from 78% to 93%, 82% to 95%, 83% to 93% and 88% to 95% respectively. They further found that the sensitivity, specificity, PPV and NPV of standard CT in detecting mediastinal staging was 57% (95%CI: 49%-66%), 82% (95%CI: 77%-86%), 56% (range, 26% to 84%) and 83% (range, 63% to 93%) respectively in a pooled analysis of 20 studies with 3438 evaluable patients.

In another study of pathologically proven NSCLC cases who underwent staging using PET/CT and CT from July 2008 to February 2012, Xu *et al*[13] concluded that PET-CT confers significantly higher accuracy than CT in nodal staging. Though PET-CT had a low sensitivity and high false-negative rate, it was shown to be more specific and accurate than CT in detecting nodal metastasis; the sensitivity, specificity, positive and negative predictive values, and accuracy of PET/CT for detecting nodal metastasis were 51.5%, 95.8%, 74.3%, 89.3% and 87.3% respectively and the corresponding data by CT were 45.5%, 87.1%, 45.5%, 87.1% and 79.2%, respectively following evaluation of a total of 528 lymph node stations in 101 patients. In a similar study of pathologically proven NSCLC cases who underwent staging using PET/CT and CT, Shim *et al*[14] also concluded that FDG PET-CT was significantly better than stand-alone CT for lung cancer staging and provided enhanced accuracy and specificity in nodal staging; they reported that the sensitivity, specificity, and accuracy of CT were 70% (23 of 33 nodal groups), 69% (248 of 360), and 69% (271 of 393) respectively, whereas those of PET/CT were 85% (28 of 33), 84% (302 of 360), and 84% (330 of 393) for the depiction of malignant nodes.

One of the major advantages of accurate loco-regional staging is to avoid futile thoracotomy. In a study to evaluate the clinical effect of PET-CT on preoperative staging of NSCLC, Fischer *et al*[15], concluded that the use of PET-CT reduced both the total number of thoracotomies and the number of futile thoracotomies, though it did not affect overall mortality.

The next natural question comes: Can PET-CT replace invasive mediastinal staging with available evidence? American College of Chest Physicians (ACCP) Evidence-Based Clinical Practice Guidelines[11] categorized intra-thoracic radiographic abnormalities into four groups based on both primary tumor and mediastinal nodes: Group A - extensive mediastinal infiltration that encircles the vessels and airways, so that the discrete lymph nodes can no longer be discerned or measured; Group B - enlargement of discrete mediastinal nodes that can be measured (> 1 cm in short-axis diameter on transverse CT image); Group C - normal mediastinal nodes determined by CT scan, but with a central tumour (within proximal one-third of hemithorax) or suspected N1 disease (enlarged N1 nodes); Group D - normal mediastinal and hilar nodes and a peripheral tumor (within outer two-thirds of the hemithorax). The ACCP guidelines recommended that radiographic (CT) assessment of the mediastinal stage is usually sufficient without invasive confirmation in group A patients as the radiographic evidence of mediastinal involvement is almost universally considered adequate in these patients. In group B and C patients, invasive staging of the mediastinum is recommended over staging by imaging alone. Invasive staging

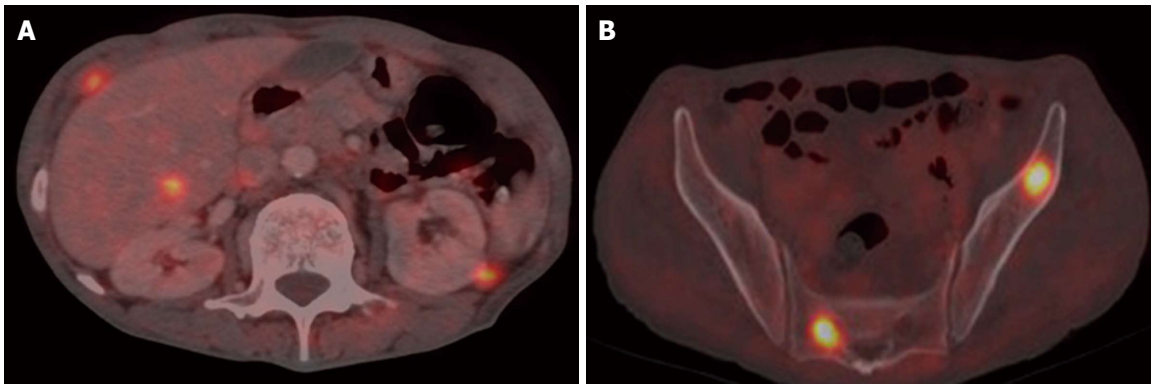

Figure 2 18-fluoro-deoxyglucose avid sub capsular hepatic deposits, liver lesion and left perinephric deposits (A) and 18-fluoro-deoxyglucose avid lesion in the sacrum and left ilium (B).

of mediastinum can be omitted in group D if PET-CT in the mediastinum is negative. The ACCP systematic review further found that the false negative rate of CT in the group of patients with T1 tumours (*i.e.*, clinical stage 1A) is approximately 9% and a negative PET-CT scan in the mediastinum carries an false negative rate of approximately 5% (range, 3% to 6%).

Another important advantage of PET-CT is identification of nodal metastasis sites which are not imaged properly with conventional imaging. Nodal stations at aorto-pulmonary window, anterior mediastinum, and posterior sub-carinal area are difficult to access on conventional imaging; FDG-PET detection of suspected metastatic nodes at these stations mandates may change the strategy of invasive mediastinal staging[3]. So, the real benefit of PET-CT is to direct the oncologist to nodal stations which need to be targeted.

Distant metastasis

Failure to identify extra-thoracic metastasis is considered as one of the important reason for poor survival in potentially curable NSCLC. This undetected metastasis causes under-staging of disease. Common sites for distant metastasis of NSCLC are brain, adrenal glands, liver, bones, kidney and abdominal lymph nodes (Figure 2).

CT scan of the chest along with upper abdomen is used for scanning the liver and adrenal glands in lung cancer. Adrenal masses are detected in approximately 20% of NSCLC cases at initial presentation. Adenomas, rather than metastasis, are used to be present in two-third of these cases. The per-cutaneous biopsy is the gold standard for confirming the status of adrenal lesions; but it is invasive and difficult to perform. A retrospective study analyzed FDG PET scans of lung cancer patients who were found to have an adrenal mass on CT or MRI scans; the sensitivity, specificity, and accuracy for detecting metastatic disease were found to be 93%, 90% and 92%, respectively following evaluation of 113 adrenal masses (75 unilateral and 19 bilateral; size range, 0.8-4.7 cm) in 94 patients[16]. The authors concluded that FDG PET was an accurate, noninvasive technique for differentiating benign from metastatic adrenal lesions detected on CT or MRI in patients with lung cancer. In another study, the depiction of adrenal gland metastasis, the sensitivity, specificity, and accuracy of PET were 74%, 73% and 74%, respectively, whereas those of integrated PET-CT were 80%, 89% and 84% respectively; thus use of PET-CT was more accurate than the use of PET alone for differentiating benign and metastatic adrenal gland lesions in lung cancer patients[17,18].

Bone scintigraphy is commonly used for detecting bone metastasis in patients with lung cancer. A meta-analysis was performed to evaluate and compare the capability for bone metastasis assessment of PET-CT, PET, MRI and bone scintigraphy in lung cancer patients found that both PET-CT and PET were better imaging methods for diagnosing bone metastasis from lung cancer than MRI and bone scintigraphy; it was concluded that PET-CT has higher diagnostic value (sensitivity, specificity and diagnostic odd ratio) for diagnosing bone metastasis from lung cancer than any other imaging methods[19].

PET-CT has low sensitivity in detecting brain metastasis due to high physiological glucose uptake by the brain cell. MRI of the brain should be used in patients with neurological symptoms to detect metastasis. FDG PET had shown better specificity in detecting liver metastasis in comparison to CECT[3].

Table 1 displays the previously published randomized controlled trials (RCTs) to assess the role of PET in the management of NSCLC[15,20-23]. The first three RCTs incorporated PET while last two RCTs included PET-CT. Three of the five RCTs concluded that use of PET leads to better disease staging which significantly decreases the futile thoracotomies; this has many ramifications including avoidance of non-curative surgery related morbidity and better utilization of health resources.

PREDICTION AND ASSESSMENT OF RESPONSE FOLLOWING NEOADJUVANT THERAPY

Multimodality treatment is the standard of care for stage

Table 1 Previously published randomized controlled trials to assess role of positron emission tomography in non-small cell lung cancer

Ref.	Publication year	Control arm	Test arm	Primary outcome	Result	Conclusion
van Tinteren *et al*[20] (PLUS study)	2002	CI ± brain imaging + invasive diagnostic procedures (n = 96)	CI ± brain imaging + PET + invasive diagnostic procedures (n = 92)	Number of futile thoracotomy	Significant reduction in futile thoracotomy with PET-CT as compared to CI (19 *vs* 39, P = 0.003, relative reduction 51%, 95%CI: 32%-80%)	Addition of PET to CI prevented unnecessary surgery in one out of five patients in suspected NSCLC
Viney *et al*[22]	2004	CI (n = 92)	CI + PET (n = 91)	Proportions of patients in whom thoracotomy was avoided	No significant reduction in thoracotomy with the use of PET as compared to conventional imaging (4 *vs* 2, P = 0.2)	PET has the potential for more appropriate stage specific therapy, it may not lead to a significant reduction in the number of thoracotomies avoided
Herder *et al*[23]	2006	CI ± brain imaging + invasive diagnostic procedures (n = 233)	CI ± brain imaging + invasive diagnostic procedures (n = 232)	Number of tests and procedures to finalize staging and operability	Equal mean (standard deviation) number of procedures to finalize staging in CI and PET arm; 7.9 (2.0) *vs* 7.9 (1.9), P = 0.90	No significant reduction in total numbers of diagnostic procedures in two groups
Fischer *et al*[15]	2009	CI + invasive diagnostic procedures (n = 91)	Conventional imaging + PET-CT + invasive diagnostic procedures (n = 98)	Number of futile thoracotomy	Reduction in futile thoracotomy with PET-CT (21 *vs* 38, P = 0.05)	PET-CT reduced both the total number of thoracotomies and the number of futile thoracotomies
Maziak *et al*[21]	2009	CI ± brain imaging + invasive diagnostic procedures (n = 167)	PET-CT + brain imaging + invasive agnostic procedures (n = 170)	Correct upstaging to avoid stage inappropriate surgery	Significantly more upstaging with PET-CT as compared to CI (13.8% *vs* 6.8%, difference 7.0%, P = 0.046)	PET-CT identifies more patients with mediastinal and extra-thoracic disease than CI

PET-CT: Positron emission tomography-computed tomography; NSCLC: Non-small cell lung cancer.

ⅢNSCLC patients. The therapeutic options available for these patients are definitive chemo-radiotherapy, or neoadjuvant therapy followed by surgical resection. Neoadjuvant therapy includes either chemotherapy or chemo-radiotherapy. Early assessment of response to neoadjuvant therapy is of paramount importance to identify non-responsive tumors; this would help in avoiding continuation of ineffective therapy and would lead to change in treatment strategy early in the course of treatment[24,25]. PET-CT has been evaluated for its multiple roles in the setting of neoadjuvant treatment; as a predictive marker for response, as a tool of assessment of response, and as a prognostic marker. The basic advantage of PET-CT in response assessment following neoadjuvant therapy is based on the premise that metabolic response precedes the morphological response[26]. However, there are many grey areas when one considers the role of PET-CT in the neoadjuvant therapy. What constitutes the metabolic response has been a real bone of contentions? What are the valid indicators for metabolic response? How much reduction of standard uptake value (SUV)max should be labeled as response following neoadjuvant therapy? What should be the interval between the pre and post therapy PET-CT. There is limited literature which is marked by the obvious heterogeneity of data: Profile of the patients, stage and histopathological types, type of chemotherapy, use of PET or integrated PET-CT, different PET-CT derived variables, and different end points for comparison. There are a few studies which have assessed the role of PET-CT in neoadjuvant setting in NSCLC; most studies included patients of both stage Ⅲ and Ⅳ NSCLC.

In a study of 34 NSCLC patients who received neoadjuvant therapy, Cerfolio *et al*[27] concluded that PET-CT had a significantly high PPV and NPV as compared to CT (81% and 94% *vs* 50% and 91% respectively for nodal disease); they defined suspicious lymph nodes on FDG-PET scans as any node with a mean SUV of greater than 3.0. Pöttgen *et al*[28] suggested that corrected SUVmax values from two serial PET-CT scans, before and after three chemotherapy cycles or later, allowed prediction of histopathological response in the primary tumor and mediastinal lymph nodes. In a prospective study of 22 patients with locally advanced NSCLC patients who had pre- and post neoadjuvant treatment PET-CT, Soussan *et al*[29] concluded that metabolic tumor volume and total lesion glycolysis ratios were the only indices correlated with residual viable tumour after induction chemotherapy; and there was no significant correlation between SUVmax and SUVmean with residual viable tumour. Kaira *et al*[30] reported that high ratio of SUVmax of the metastatic tumor to the primary tumor (M/P ratio) was associated with a poor response to initial chemotherapy. In a prospective multicenter study of 47 stage ⅢA-N2 NSCLC patients who were imaged with PET before the start of platinum-based induction chemotherapy, after the first cycle, and within 3 to 4 wk after completion of the third cycle, Hoekstra *et al*[31] reported that a 35% decrease of FDG uptake discriminated responders from

non-responders (P = 0.03). Prognostic value of PET-CT has also been addressed in the management of NSCLC. In a retrospective evaluation of 205 stage ⅢA NSCLC patients who underwent surgical resection after neoadjuvant chemo-radiotherapy, Lee *et al*[32] concluded that SUVmax was a sole independent factor for survival in multivariate analysis in whole series (SUVmax cutoff, 13; median survival, 3.0 years *vs* 4.0 years; P = 0.016).

The current review illustrates that there is high heterogeneity in various studies with respect to patient profile, methods of measurement of FDG uptake, timing with respect to anticancer therapy, and different thresholds to define metabolic response; further studies which exclusively include stage Ⅲ NSCLC patients are required to draw definite conclusions on PET-CT as a tool for neoadjuvant therapy response monitoring.

CONCLUSION

The role of PET-CT in the management of non-small cell lung cancer continues to emerge with time. Besides better loco-regional and distant staging of disease in one sitting, detection of abnormal mediastinal nodes at various basins for better targeted biopsy is the potential advantage of PET-CT and may lead to reduction in futile surgical interventions. This has made PET-CT an essential component in the initial staging of patients with NSCLC. The role of PET-CT in the prediction and assessment of response to neoadjuvant therapy needs further studies.

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MINIREVIEWS

Potential effects of curcumin on peroxisome proliferator-activated receptor-γ *in vitro* and *in vivo*

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Abstract

Natural peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists are found in food and may be important for health through their anti-inflammatory properties. Curcumin (Cur) is a bright yellow spice, derived from the rhizome of Curcuma longa Linn. It has been shown to have many biological properties that appear to operate through diverse mechanisms. Some of these potentially beneficial effects of Cur are due to activation of the nuclear transcription factor PPAR-γ. It is reported (using *in vitro* and *in vivo* models) that Cur plays a potential role against several diseases. In this review article, we present the current literature on the effects of Cur on the modulation of inflammatory processes that are mediated through PPAR-γ.

Key words: Curcumin; Anti-inflammatory; Peroxisome proliferator-activated receptor-γ

Core tip: In this short review, we highlight the potential antioxidant and anti-inflammatory properties of curcumin (Cur), discussing its impact on peroxisome proliferator-activated receptor-γ (PPAR-γ) receptor function and its effects *in vitro* and *in vivo*. Cur affects the

PPAR-γ gene and prevents cell growth through effects on the cell cycle and induction of apoptosis. It is also well-established that Cur has anti-inflammatory effects *in vivo* through regulation of the PPAR-γ receptor, which leads to the suppression of nuclear factor kappa B, a pro-inflammatory mediator.

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INTRODUCTION

Curcumin

Curcumin (diferuloylmethane) (Cur) is an orange pigment extractable from turmeric. Curcuma is derived from the word "Kourkoum". Due to its color, curcuma is sometimes referred to in Europe as "Indian Saffron". As a result of its chemical and biological properties, Cur is known to contain several potential important phytochemical compounds[1-5]. Cur is a lipophilic polyphenol, is poorly soluble in water and stable at an acidic pH[6]. A critical review of Cur suggests that the compound has potential as a modulator of the activity of many vital bio-macromolecular targets involved in homeostasis of mammalian physiology[7]. Dietary polyphenols have recently received more attention because of their potentially protective characteristics against metabolic diseases[8].

The properties of Cur

Cur has been reported to be safe at dosages of up to 8 g/d in human studies and there is no evidence of resistance. Nevertheless, bioavailability is a major concern as 75% of Cur is excreted in the stool[9,10]. Besides its dietary use, Cur has been considered to have beneficial properties, including anti-inflammatory, antioxidant, antineoplastic, pro and anti-apoptotic, anti-angiogenic, cytotoxic, immune-modulatory and antimicrobial effects, through the modulation of various kinds of targets, including growth factors, enzymes and genes such as *STAT3*, peroxisome proliferator-activated receptor-γ (PPAR-γ) and nuclear factor kappa B (NF-κB)[11,12]. It also has a strong anti-inflammatory effect that inhibits several mediators of the inflammatory response[13-15]. Due to its low solubility in water and therefore poor oral bioavailability, nanoparticles and liposomes have been suggested as potential ways of improving its efficacy[16].

PPARs

PPARs are a class of proteins that are usually activated by their respective ligands and function within the cell nuclei for controlling metabolism, development and homeostasis. PPARs heterodimerize with the retinoid X receptor and bind to PPAR responsive element in the regulatory region of target genes that function in different natural courses, such as adipogenesis, immune response and both cell growth and differentiation[17,18]. There are 3 major isoforms of PPARs in mammals, namely PPARα, PPAR-γ and PPARα/γ. PPAR-α can improve triglyceride concentration and also has some roles in energy homeostasis, whereas activation of PPAR-α/γ improves fatty acid hemostasis[19]. PPAR-γ is involved in lipid anabolism, adipocyte differentiation inflammation and immune response[20]. PPAR-α is triggered by a wide diversity of fatty acids or their metabolites and governs metabolic processes implicated in glucose and lipid metabolism and adipose mass control by modulating the expression of a huge quantity of target genes. Furthermore, PPAR-γ is a molecular target for anti-diabetic thiazolidinedione molecules that selectively bind this nuclear receptor to improve systemic insulin sensitivity and glucose tolerance. Accordingly, the specific position of PPAR-γ in systemic metabolic control is due to its pivotal role in the homeostasis control of glucose and lipid homeostasis, lipid storage and adipogenesis[21]. Lately, PPAR-γ has been recognized to be the major player with a key role in the immune response because of its capability to prevent the production of inflammatory substances[22].

Hepatic stellate cells and liver fibrosis

Hepatic stellate cells (HSCs) are located near to hepatic epithelial cells. In a normal liver, HSCs contain many vitamin A lipid droplets. When the liver is injured, HSCs receive signals from damaged cells in the liver to change into activated myofibroblast-like cells[23,24]. In addition, HSCs secrete growth factors and help in the maintenance of liver cells. In liver disease, extended and frequent activation of HSCs causes liver fibrosis that may eventually result in organ failure and death[25,26]. Activation of hepatic HSCs is a key step in liver collagen production and fibrosis formation[27-31]. Hepatic fibrosis is also a necessary step in the development of hepatic cirrhosis. Thus, treatment of chronic liver diseases depends on the prevention and treatment of fibrosis[32]. Some studies showed that HSC activation significantly reduces the expression of PPAR-γ and that PPAR-γ agonists inhibit HSC activation, resulting in reduced expression of α-SMA and collagen, as well as reduced cell propagation and development of hepatic fibrosis. In normal liver tissues, PPAR-γ is expressed highly in quiescent HSCs. Moreover, increased PPAR-γ expression reduces the synthesis of HSC DNA and results in the diminished expression of collagen and the transforming growth factor (TGF)-1β. At the same time, PPAR-γ is also involved in the apoptosis of HSCs through a variety of mechanisms[33-36]. Some experiments have confirmed that Cur may prevent the proliferation of HSCs whilst also increasing their apoptosis[37]. A further study has shown that Cur increases the expression of PPAR-γ and revives the trans-activating activity in activated

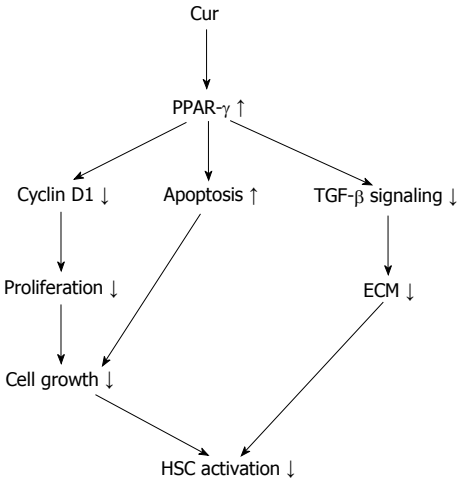

Figure 1 Possible mechanisms, primarily the inhibition of hepatic stellate cell activation by peroxisome proliferator-activated receptor-γ after modulation with curcumin. PPAR-γ: Peroxisome proliferator-activated receptor-γ; HSC: Hepatic stellate cell; TGF: Transforming growth factor; Cur: Curcumin; ECM: Extracellular matrix.

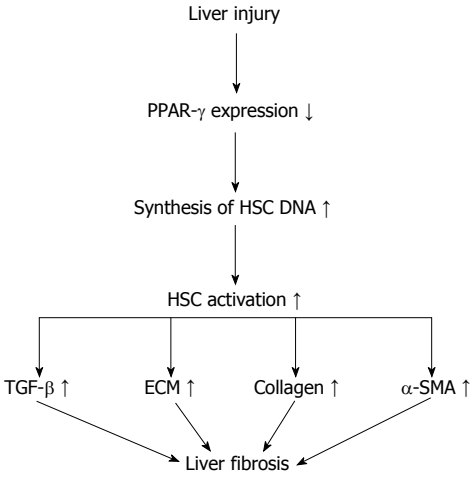

Figure 2 Liver fibrosis creation followed down-regulating of peroxisome proliferator-activated receptor-γ after liver injury. As shown, decrease in PPAR-γ expression after liver injury causes an increase in HSC DNA expression and HSC activation. This regulation also results in increased expression of α-SMA, collagen, ECM and TGF-β and induces liver fibrosis. PPAR-γ: Peroxisome proliferator-activated receptor-γ; HSC: Hepatic stellate cell; TGF: Transforming growth factor; ECM: Extracellular matrix; α-SMA: α-smooth muscle actin.

HSC, which is essential for the anti-inflammatory and antioxidant effects on reserve for HSC propagation and growth[38] (Figure 1).

In this review article, we present the current literature to display the role of Cur on modulation of inflammatory processes that are mediated through PPAR-γ.

EFFECTS OF CUR ON PPAR-γ EXPRESSION IN HSCS AND HEPATIC FIBROSIS

HSCs are activated when gene expression and phenotype changes render the quiescent cells responsive to other cytokines. Kupffer cells provide the potential source of paracrine stimuli for HSCs because they express TGF-β[24,25,39-41]. During HSC activation, regulatory pathways including epigenetic regulation of (NF-κB) and reduction in PPAR-γ expression modulate the expression of many genes, including *TGF-1β* and *MMP-2*[42-46].

Many *in vitro* studies have shown that Cur inhibits cell proliferation and induces apoptosis of stimulated HSC. However, the mechanism and action of Cur on HSC growth *in vitro* is not well defined. Numerous mechanisms have been recognized for the inhibition of TGF-1β signaling *via* Cur, including PPAR-γ activation. Cur inhibits NF-κB, leptin and insulin and mediates HSC activation by stimulating PPAR-γ activity[38,47-51] (Figure 2).

Zheng *et al*[52] confirmed that inhibiting PPAR-γ stimulation abrogated the effects of Cur on the stimulation of apoptosis and prevention of the expression of *ECM* genes in activated HSC *in vitro*. They also showed that Cur repressed the gene expression of TGF-β receptors and disturbed the TGF-β signaling pathway in stimulated HSC, which is facilitated by PPAR-γ stimulation[52]. Zhang *et al*[37] established that Cur improved fibrotic injury and sinusoidal angiogenesis in the rodent liver when fibrosis was initiated by carbon tetrachloride. Cur decreased the expression of a number of angiogenic factors in the fibrotic liver. Moreover, *in vitro* investigation showed that the sustainability and vascularization of rodent liver sinusoidal endothelial cells and angiogenesis in rodents were not diminished by Cur. These findings demonstrated that HSCs could be a possible target for Cur. Moreover, other studies have shown that Cur can inhibit vascular endothelial growth factor expression in HSCs associated with interrupting the mammalian target of rapamycin pathway. PPAR-γ activation was reported to be essential for Cur to prevent the angiogenesis in HSCs. The authors determined that Cur reduced sinusoidal angiogenesis in liver fibrosis probably by HSCs *via* a PPAR-γ activation-dependent pathway. Also, other studies showed that PPAR-γ could be a target molecule for decreasing pathological angiogenesis in liver fibrosis for rodents[37]. These studies offer new perspectives into the mechanisms that underpin prevention of HSC activation by Cur and PPAR-γ ligands and inhibit HSC activation and liver fibrosis. To convert stimulated HSCs to a quiescent state or to induce apoptosis may be a dangerous approach for anti-fibrotic treatment.

EVIDENCE FOR THE PPAR-γ MEDIATED ANTI-INFLAMMATORY EFFECT OF CUR

It appears that the hydroxyl and methoxy residues of Cur are accountable for its antioxidant and anti-inflammatory effects[53,54]. Some of the effects of Cur are through the JAK/STAT pathway, which can decrease pro-inflammatory interleukins and cytokines. Moreover, Cur

Table 1 Molecular targets of curcumin and peroxisome proliferator-activated receptor-γ modulated by curcumin *in vivo* and *in vitro*

Transcription factors	Growth factor/or cytokines	Proteins/or protein kinase pathway	Inflammatory mediators	Enzymes
STAT3 ↓	TGF-β ↓	Cyclin D1 ↓	IL-1 ↓	LOX ↓
NF-κB ↓	TNF-α ↓	Collagen ↓	IL-2 ↓	XO ↓
	MCP-1 ↓	LDL ↓	IL-6 ↓	COX-2 ↓
		Insulin ↓	IL-8 ↓	iNOS ↓
		Leptin ↓	LOX ↓	
		JAK/STAT ↓		

NF-κB: Nuclear factor kappa B; TGF: Transforming growth factor; LDL: Low-density lipoprotein; LOX: Lipoxygenase; COX: Cyclooxygenase; STAT3: Signal transducer and activator of transcription 3; TNF: Tumor necrosis factors; MCP-1: Monocyte chemoattractant protein-1; IL: Interleukin; iNOS: Inducible nitric oxide synthase; XO: Xanthine oxidase.

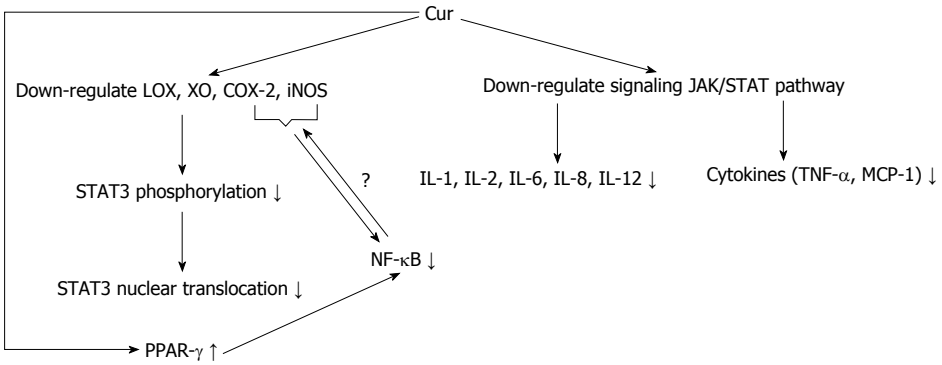

Figure 3 Mechanisms of anti-inflammatory properties of curcumin *in vivo*. Curcumin (Cur) down-regulates some of the factors involved in inflammation, inhibiting NF-κB activation and causing its anti-inflammatory effects. Also, Cur with increasing PPAR-γ expression directly inhibits NF-κB activation. NF-κB: Nuclear factor kappa B; TNF: Tumor necrosis factors; MCP-1: Monocyte chemoattractant protein-1; IL: Interleukins; LOX: Lipoxygenase; COX: Cyclooxygenase; iNOS: Inducible nitric oxide synthase; STAT3: Signal transducer and activator of transcription 3; PPAR-γ: Peroxisome proliferator-activated receptor-γ; XO: Xanthine oxidase.

suppresses the inflammatory response by decreasing the activity of cyclooxygenase-2 (COX-2) and lipoxygenase, resulting in inhibition of STAT3 phosphorylation and consequent STAT3 nuclear translocation[55-58]. Cur suppression of COX-2 and inducible nitric oxide synthase may be *via* the inhibition of the NF-κB activation by this polyphenol group.

Kawamori *et al*[59] have shown that dietary Cur inhibits phospholipase A2 and affects COX and lipoxygenase actions. Cur decreases COX-2 expression at the transcriptional level[13]. Cur is supposed to inhibit NF-κB and pro-inflammatory substances by hindering phosphorylation of inhibitory factor Ⅰ kappa B kinase. The growing incidence of allergic disease, combined with promising outcomes from RCTs, proposes that natural PPAR-γ agonists found in the diet might be helpful by acting as anti-inflammatory factors[59-61].

Cur has been reported to trigger PPAR-γ but whether or not it is a ligand for it is still debated and further experimental work is required in this regard (Figure 3). Moreover, the exact mechanisms by which Cur stimulates PPAR-γ expression are still unknown. Given the important role of Cur, there may be two ways. Cur binds to its own receptor and the complex stimulates the up-regulation of PPAR-γ, or Cur is a ligand of PPAR-γ leading to the stimulation of PPAR-γ[62,63]. A summary of the possible molecular targeting of Cur and PPAR-γ modulated by Cur is shown in Table 1. Investigators have described the *in vitro* anti-inflammatory pathways of Cur and they suggest that it was reached mostly through the down-regulation of NF-κB[4,16]. Most experiments have shown that the anti-inflammatory effect of Cur is attributed to PPAR-γ activation[64]. Recent experimental data have shown that Cur has an antitumor effect in pancreatic cancer by inhibiting propagation and down-regulating NF-κB and its products[65]. Nevertheless, it is reasonable to suggest that Cur prompted an anti-inflammatory effect through the up-regulation of PPAR-γ which is closely related to the NF-κB pathway.

CONCLUSION

In this short review, we have highlighted the potential antioxidant and anti-inflammatory activities of Cur and discussed Cur's significant impact on PPAR-γ receptor function. Cur prompts the expression of the *PPAR-γ* gene, causing its activation in cells to activate HSCs and hepatic fibrosis. This combined action of Cur and PPAR-γ prevents cell growth from the stimulation of the cell cycle and induction of apoptosis. It is also well-established that Cur has anti-inflammatory effects *in vivo* through regulation of the PPAR-γ receptor, which leads to the suppression of NF-κB, a pro-inflammatory mediator.

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Observational Study

Profile and determinants of unsuccessful tuberculosis outcome in rural Nigeria: Implications for tuberculosis control

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at ukwajakingsley@yahoo.co.uk.

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Abstract

AIM: To determine the treatment outcomes and predictors for unsuccessful tuberculosis (TB) outcomes in rural Nigeria.

METHODS: Adult rural TB patients treated during 2011 and 2012 in two healthcare facilities (one urban public and one rural private) were identified from the TB treatment registers and retrospectively reviewed. Tuberculosis treatment outcomes were assessed according to World Health Organisation guidelines. Determinants of unsuccessful treatment outcomes were identified using a multivariable logistic regression analysis.

RESULTS: Between January 2011 to December 2012, 1180 rural TB patients started treatment, of whom 494 (41.9%) were female. The treatment success rate was 893 (75.7%), while the rates of death, loss-to-follow-up, and treatment failure were 129 (10.9%), 100 (8.5%), and 18 (1.5%) respectively. In the final multivariable logistic regression model, the odds of unsuccessful treatment outcome were higher among patients who received care at the urban public facility (aOR = 2.9, 95%CI: 1.9-4.4), smear-negative (1.3,

1.0-1.8) and extrapulmonary (2.7, 1.3-5.6) TB patients, human immunodeficiency virus (HIV) co-infected (2.1, 1.5-3.0), and patient who received the longer (8-mo) anti-TB regimen (1.3, 1.1-1.8).

CONCLUSION: Treatment success among rural TB patient in Nigeria is low. High risk groups should be targeted for closer monitoring, socio-economic support, and expansion of TB/HIV activities.

Key words: Tuberculosis; Treatment outcome; Rural; Health services; Nigeria

Core tip: Of 1180 tuberculosis (TB) patients enrolled, overall treatment success rate was 893 (75.7%). Also, death, default, and treatment failure rates were 10.9%, 8.5%, and 1.5%, respectively. Treatment success rate were significantly higher among all human immunodeficiency virus (HIV)-negative TB cases (79.3% *vs* 60.9%; $P < 0.001$). The difference was due to higher death rates among HIV-infected TB patients. Predictors of unsuccessful outcomes were; public facility-care, smear-negative or extrapulmonary TB, HIV co-infection, and receiving the 8-mo regimen.

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INTRODUCTION

Despite recent progress in tuberculosis (TB) control, the disease is still a leading cause of mortality globally and a major public health challenge in low- and middle-income countries[1]. Adverse outcomes of TB tend to be high in poor populations[2]. As poverty is both a cause and outcome of TB, it is closely associated with the socioeconomic status of a population - and it remains a major driver of the disease-poverty trap seen in underserved populations[2]. Rural residence can be a marker for poverty and, thus vulnerability to TB[3]. Previous studies demonstrate that rural TB patients often do not recognise TB symptoms[4]. This have resulted in prolonged delays in seeking care for TB[4,5], and may lead to increased risk of continuing community TB transmission and important burden of undiagnosed active TB in the community[6]. Moreover, when these rural patients eventually present to health services, they arrive with more advanced TB disease[7]. Furthermore, a qualitative study suggests that rural TB are less likely to complete TB treatment due to being unaware of the duration of TB treatment, stopping treatment once symptoms subsided, and lack of family support[8]. Thus, addressing poverty in TB control should include not only the needs of those facing economic impoverishment but also all relatively vulnerable, disadvantaged, marginalized sections of the population like TB patients residing in rural areas[2,3,5].

Although the expansion of TB treatment services and coverage through the directly observed treatment short course (DOTS) strategy have resulted in considerable progress in TB control in high TB burden countries[9], there is limited information on outcomes of TB treatment in underserved populations in those settings. Assessing the outcomes of TB treatment is essential for the evaluation of the effectiveness of the DOTS services[1,10]. Furthermore, identifying the specific determinants for unsuccessful outcomes is important to design interventions that would improve treatment systems[9,11]. As addressing the needs of TB among the poor and vulnerable populations is one of the key components of the StopTB strategy[12], information on the treatment outcomes and the determinants of adverse outcomes in rural TB patients is crucial. To date, only few studies have evaluated the outcomes of TB among rural TB patients[10,13-17], and far less have documented independent risk factors for poor treatment outcomes among them in high-burden settings[15,16].

More than 70% of Nigerians live in the rural area where up to 80% of the population lives below poverty line, and public healthcare services are hardly available in this setting in Nigeria[18,19]. Also, a high proportion of rural TB patients in Nigeria face substantial patient and health systems delays before reaching an appropriate health care provider[5]. Poor care in the TB care-seeking pathway increases the costs that already impoverished individuals and families encounter and commonly results in TB patients being unable to work for long periods while at the same time incurring catastrophic costs[19,20]. Thus, there is a need to assess the performance of the tuberculosis control programme in rural Nigeria in order inform health policy solutions crucial for improving programme performance. The aim of this study was to investigate the treatment outcomes of TB and the determinants of adverse outcomes in rural Nigeria.

MATERIALS AND METHODS

Study design

This was a retrospective observational study using routine programme data. The study was part of large operational research project to evaluate the profile and treatment outcomes of subpopulations of TB patients as well as identify possible determinants of unsuccessful outcomes in these patients in order to highlight areas for priority intervention for the TB control programme in Ebonyi State[21-23]. The present study population consisted of all adult (≥ 15 years) TB patients treated between 1 January 2011 and 31 December 2012 who were recorded in the TB treatment registers as resident

in a rural area.

Study area

The study was carried out in Ebonyi state Southeastern Nigeria. Ebonyi state has a population of over 2.5 million people, 75% of them resides in the rural area[18]; and 74% live below the poverty line[24]. TB notification rate for new cases was 77/100000 in 2009[25]. Due to health system gaps, there is limited availability of public facilities in rural settings in the state[19,20]. Thus, several mission (private) hospitals give primary and secondary care services in those settings. Two hospitals - one rural secondary-care (faith-based/mission) private and the one only tertiary-care (urban/public) hospital in the state were selected as the study sites due to their geographic spread and high TB notification rate. Both hospitals accounts for about 50% of annual TB notification in Ebonyi State[25]; and receives referrals from nearby states in Southern Nigeria.

Diagnosis of TB

Any person with a cough lasting for two or more weeks with or without weight loss, night sweats, fever, and shortness of breath were evaluated for TB. Three sputum specimens were submitted for light microscopy using Ziehl-Neelsen staining methods. The presence of acid-fast bacilli (AFB) in one or more sputum samples in a patient qualifies as a case of smear-positive pulmonary TB (SPTB)[26]. Patients with smear-negative sputum are given broad spectrum antibiotics and further evaluated using clinical assessments, radiographs, and repeat sputum examinations for AFB before the diagnosis of smear-negative TB (SNTB) is made. Extrapulmonary TB (EPTB) is diagnosed on the basis of clinical/laboratory evidence and a decision by an experienced medical officer[26]. TB/human immunodeficiency virus (HIV) collaborative activities exist in all the study facilities; therefore, all TB patients are counselled and tested for HIV, and vice versa[26].

Tuberculosis treatment

The treatment was based on the community DOTS strategy - where intake of the anti-TB medications is being observed daily by a DOTS-supporter or a community health worker residing in the same community as the patient. The intensive phase of treatment lasted for two months for new patients and three months for retreatment patients. Before 2012, all new pulmonary tuberculosis patients were treated using an eight-month anti-tuberculosis regimen consisting of two months of intensive phase treatment with rifampicin (R) and isoniazid (H), pyrazinamide (Z) and ethambutol (E); and six months of isoniazid and ethambutol, *i.e.,* 2RHZE/6EH[26,27]. However, from January 2012, the regimen was changed to a six-month regimen containing 6 mo of rifampicin (2RHZE/4RH) in line with the recent World Health Organization (WHO) guidelines[27]. All retreatment cases received a 3-mo intensive phase with the addition of streptomycin to RHZE in the first two months; and a continuation phase of 5RHE[26,27]. During the intensive phase of treatment, medications were collected twice a month; afterwards, medications were collected monthly. Fixed-dose combinations of anti-TB drugs were used[26].

Definitions of TB treatment outcomes

We used the standard WHO definitions of TB treatment outcomes[1,26,27]. Briefly these include: Cured (a patient who was initially smear-positive and who was smear-negative in the last month of treatment and on at least one previous occasion), completed treatment (a patient who completed treatment, but who did not meet the criteria for cure or failure - this definition applies to smear-positive and smear-negative patients and to patients with EPTB), death (a patient who died from any cause during the course of treatment), treatment failure (a patient who was initially smear-positive and who remained smear positive at month 5 of treatment or later during treatment), lost to follow-up (a patient whose treatment was interrupted for 2 consecutive months or longer), and transferred-out (a patient who transferred to another reporting unit and for whom treatment outcome is unknown). A successfully treated individual is a patient who was cured or who completed treatment.

Data collection and variables

Variables retrieved from the TB treatment registers were related to the study objectives. Patients' age, gender, facility (public *vs* private), type of TB, treatment category, treatment regimen (six-month *vs* eight-month) and HIV-status were the main explanatory variables. In addition to standard outcome definitions, we classified the final treatment outcome as a dichotomous variable, *i.e.,* successful (cured or treatment completed) *vs* unsuccessful (death, loss to follow-up, failure or transferred-out) outcomes.

Sample size

The sample size was calculated using Win Episcope 2.0. With a sample size of at least 246 patients, we were able to detect an 80% prevalence of successful outcomes[28], at 95% confidence level and an absolute sampling error of 0.05.

Statistical analysis

The data were double-entered, checked, and analyzed using Epi Info 3.4.1 (CDC, Atlanta, GA United States). Treatment outcomes were expressed as proportions (%). OR and their 95%CIs were estimated using multivariable logistic regression analysis, with treatment outcome (successful *vs* unsuccessful) as the outcome variable. The likelihood ratio test was used to assess the association between explanatory variables and outcome variable. A stratified analysis was conducted to determine the occurrence of interaction and confounding between the main outcome variable and exposure

Table 1 Demographic and clinical profile of rural tuberculosis patients, Nigeria, 2011-2012

Variables	*n* (%)
Age (yr)	
≤ 40	728 (61.7)
> 40	452 (38.3)
Gender	
Female	494 (41.9)
Male	686 (58.1)
Facility	
Private	1035 (87.7)
Public	145 (12.3)
Treatment category	
New	1099 (93.1)
Retreatment	81 (6.9)
Type of TB	
Pulmonary TB	1134 (96.1)
Extrapulmonary TB	46 (3.9)
HIV status	
HIV - positive	233 (19.7)
HIV - negative	947 (80.3)
Treatment regimen (*n* = 1099)	
Regimen 1	579 (52.7)
Regimen 2	520 (47.3)

Regimen 1: 8-mo regimen; Regimen 2: 6-mo regimen. TB: Tuberculosis; HIV: Human immunodeficiency virus.

variables. A multivariable logistic regression model was then constructed using the full model fits. $P < 0.05$ was considered statistically significant.

The statistical methods of this study were reviewed by Femi Gbenga from Femo Stat Consult, Abakaliki, Ebonyi State, Nigeria.

RESULTS

Socio-demographic characteristics

A total of 1180 rural TB patients were treated during the study period; of whom 494 (41.9%) were female. Majority of the patients 1099 (93.1%) had newly diagnosed TB while 81 (6.9%) were retreatment cases. The mean ± SD age of all patients was 39.3 ± 15.1 years. Also, 708 (60%) of them had smear-positive pulmonary TB, 426 (36.1%) had smear-negative pulmonary TB and 46 (3.9%) had extrapulmonary TB. Furthermore, 1035 (87.7%) of all the patients were treated at the private faith-based (rural) health facility; 233 (19.7%) were HIV-positive, and 520 (47.3) were treated using the shorter six-month regimen. Table 1 shows the demographic and clinical characteristics of all patients included in the study.

Treatment outcomes

Treatment outcomes by type and category of TB are shown in Table 2. Among all TB cases seen during the study period, the treatment success rate was 893 (75.7%), while the rates of death, loss-to-follow-up, and treatment failure were 129 (10.9%), 100 (8.5%), and 18 (1.5%), respectively (Table 2). For SPTB cases (n = 708), the overall treatment success rate was 572 (80.8%); while unsuccessful outcomes were due to loss-to-follow-up 66 (9.3%), deaths 46 (6.5%), treatment failure 18 (2.5%), and transfer-out 6 (0.8%). For SNTB cases (n = 426), treatment success rate was 303 (71.7%); and unsuccessful outcomes were due to death 75 (17.6%), loss-to-follow-up 22 (5.2%), and transfer-out 26 (6.1%). Among EPTB cases (n = 46), treatment success rate was 18 (39.1%); with loss-to-follow-up 12 (26.1%) accounting for most of the unsuccessful outcomes (Table 2). Also, treatment success rate was 832 (75.7%) among new cases compared with 61 (75.3%) among retreatment cases; $P = 0.9$ (Table 2).

Furthermore, comparing HIV-negative *vs* HIV-infected TB patients (Table 2), treatment success rates were significantly higher among all HIV-negative TB cases compared to all HIV co-infected cases (79.3% *vs* 60.9%, $P < 0.001$). The difference was accounted for mainly by higher death rates among HIV-infected TB patients (23.2% *vs* 7.9%, $P < 0.001$). Also, in pulmonary TB and new TB cases, treatment success rates were higher in HIV-negative compared to HIV co-infected cases (81% *vs* 61.8%, $P < 0.001$) and (79.4% *vs* 60.8%, $P < 0.001$), respectively. And, in both cases, this was mainly due to a significantly higher death rate among HIV-infected TB patients ($P < 0.001$; Table 2). In EPTB or re-treatment TB cases, there were no significant differences in treatment success rates according to HIV status ($P > 0.05$; Table 2).

Of the 708 SPTB patients, 665 (93.9%) had a sputum AFB microscopy done at the end of the second month of treatment. From these 665 patients, 144 (21.7%) had persistent smear positive smears; while 521 (78.3%) had a negative smear conversion after the first two months of treatment. Also, 607/665 (91.3%) of the patients who had a smear test after intensive treatment had sputum AFB result at the end of the fifth month of treatment with 17 (2.8%) still being smear positive.

Determinants of unsuccessful outcomes

Univariate and multivariable logistic regression analysis was performed to determine socio-demographic and clinical risk factors for unsuccessful outcomes (Table 3). The independent predictors for unsuccessful outcomes were: Type of facility, type of TB, HIV status and treatment regimen. The risk of unsuccessful outcomes was 2.9 (1.9-4.4) times higher among TB patients who received care in the urban public facility compared to those treated at the rural private hospital. Compared to smear-positive TB patients, smear-negative and extrapulmonary TB patients were 1.3 (1.0-1.8) and 2.7 (1.3-5.6) times more likely to have unsuccessful outcomes respectively. HIV-positive TB patients had 2.1 (1.5-3.0) times greater risk of unsuccessful outcomes compared to HIV-negative patients. Unsuccessful treatment outcomes was 1.3 (1.1-1.8) times more frequent in patients who received the longer (eight-month) regimen than among those treated with the shorter (six-month) regimen (Table 3).

Table 2 Tuberculosis treatment outcomes stratified by human immunodeficiency virus status in rural Ebonyi, Nigeria, 2011-2012

Treatment outcome	Both HIV- and HIV+ *n* (%)	HIV-negative *n* (%)	HIV-positive *n* (%)	χ^2 (*P* value)
All TB cases				34.2 (< 0.001)
Successful	893 (75.7)	751 (79.3)	142 (60.9)	
Unsuccessful	287 (24.3)	196 (20.7)	91 (39.1)	
Failure	18 (1.5)	13 (1.4)	5 (2.1)	1.84 (0.18)
Death	129 (10.9)	75 (7.9)	54 (23.2)	49.0 (< 0.001)
Default	100 (8.5)	79 (8.3)	21 (9.0)	1.70 (0.19)
Transfer-out	40 (3.4)	29 (3.1)	11 (4.7)	3.76 (0.05)
Total	1180	947	233	
Pulmonary TB				37.7 (< 0.001)
Successful	875 (77.2)	736 (81.0)	139 (61.8)	
Unsuccessful	259 (22.8)	173 (19.0)	86 (38.2)	
Failure	18 (1.6)	13 (1.4)	5 (2.2)	1.84 (0.18)
Death	121 (10.7)	69 (7.6)	52 (23.1)	50.3 (< 0.001)
Default	88 (7.8)	69 (7.6)	19 (8.4)	1.9 (0.17)
Transfer-out	32 (2.8)	22 (2.4)	10 (4.4)	5.3 (0.02)
Total	1134	909	225	
Extrapulmonary TB				0.01 (0.47)[1]
Successful	18 (39.1)	15 (39.5)	3 (37.5)	
Unsuccessful	28 (60.9)	23 (60.5)	5 (62.5)	
Failure	0	0 (0)	0 (0)	-
Death	8 (17.4)	6 (15.8)	2 (25.0)	0.25 (0.5)[1]
Default	12 (26.1)	10 (26.3)	2 (25.0)	0.0 (0.68)[1]
Transfer-out	8 (17.4)	7 (18.4)	1 (12.5)	0.07 (0.64)[1]
Total	46	38	8	
New cases				32.5 (< 0.001)
Successful	832 (75.7)	700 (79.4)	132 (60.8)	
Unsuccessful	267 (24.3)	132 (20.6)	85 (39.2)	
Failure	15 (1.4)	11 (1.2)	4 (1.4)	1.28 (0.26)
Death	118 (10.7)	67 (7.6)	51 (23.5)	49.7 (< 0.001)
Default	97 (8.8)	76 (8.6)	21 (9.7)	2.1 (0.15)
Transfer-out	37 (3.4)	28 (3.2)	9 (4.1)	1.86 (0.17)
Total	1099	882	217	
Retreatment cases				1.8 (0.10)[1]
Successful	61 (75.3)	51 (78.5)	10 (62.5)	
Unsuccessful	20 (24.7)	14 (21.5)	6 (37.5)	
Failure	3 (3.7)	2 (3.1)	1 (6.3)	0.58 (0.44)[1]
Death	11 (13.6)	8 (12.3)	3 (18.8)	0.75 (0.31)[1]
Default	3 (3.7)	3 (4.6)	0 (0)	0.58 (0.59)[1]
Transfer-out	3 (3.7)	1 (1.5)	2 (12.5)	4.74 (0.08)[1]
Total	81	65	16	

[1]Fisher's exact *P*-value was reported; TB: Tuberculosis; HIV: Human immunodeficiency virus; HIV+: HIV-positive; HIV-: HIV-negative.

DISCUSSION

In order to improve TB control in an underserved population, we assessed the treatment outcomes of adult TB patients living in rural Ebonyi State, Nigeria. The study showed that treatment success rate was below recommended target and the current national levels; and was associated with the type of facility where treatment was given, type of TB, HIV status and treatment regimen received. The treatment success rate observed among rural TB patients was lower than the national TB programme and WHO target of 85%[1]. By 2011, both Nigeria and Ebonyi State had reached the WHO target treatment success rate of 85% among all TB patients[1,28]. Our finding of a success rate of 75.7% in rural patients and even lower rates in the various subgroups of TB patients studied suggests that figures reported nationally and locally are likely to mask lower treatment success levels in underserved rural populations. It also suggests that achieving the treatment success target for rural TB patients is a major challenge that needs to be tackled. Our finding agrees with other studies that evaluated outcomes of TB treatment in rural settings where treatment success rates for Angola (66.3%)[13], China (74.5%)[14] and Ghana (60.7%)[29] were all found to be below recommended target. However, other studies showed that in rural Ethiopia, Haiti, and Hunan, China TB treatment success rates were over 85%[10,16,29]. Our finding suggests the need to improve education, monitoring of cases and quality of TB management in rural settings of Nigeria.

In this study we have shown that receiving treatment at the public facility was a predictor of unsuccessful outcomes among rural TB patients. This may be due to difficulty in accessing care at the urban

Table 3 Multivariable logistic regression analysis of factors associated unsuccessful treatment outcomes among rural tuberculosis patients, Nigeria, 2011-2012

Variables	*n* = 1180 *n* (%)	Unsuccessful outcomes *n* (%)	Crude OR (95%CI)	Adjusted OR (95%CI)	Adjusted *P*-value
Total	1180	287 (24.3)			
Age (yr)					
≤ 40	728	168 (23.1)	1		
> 40	452	119 (26.3)	1.2 (0.9-1.6)	1.3 (1.0-1.8)	0.08
Gender					
Female	494	115 (23.3)	1		
Male	696	172 (25.1)	1.1 (0.8-1.5)	1.2 (0.9-1.6)	0.25
Facility					
Private	1035	210 (20.3)	1		
Public	145	77 (53.1)	4.5 (3.1-6.4)	2.9 (1.9-4.4)	< 0.001
Type of TB					
PTB smear positive	708	136 (19.2)	1		
PTB smear negative	426	123 (28.9)	1.5 (1.1-1.9)	1.3 (1.0-1.8)	0.09
Extrapulmonary	46	28 (60.9)	5.3 (2.9-9.7)	2.7 (1.3-5.6)	0.009
Treatment category					
New	1099	267 (24.3)	1		
Retreatment	81	20 (24.7)	1.0 (0.6-1.7)	1.1 (0.6-1.9)	0.75
Regimen (*n* = 1099)					
Regimen 1	579	157 (27.1)	1.4 (1.1-1.8)	1.3 (1.1-1.8)	0.04
Regimen 2	520	110 (21.2)			
HIV status					
Negative	947	196 (20.7)	1		
Positive	233	91 (31.7)	2.5 (1.8-3.3)	2.1 (1.5-3.0)	< 0.001

Regimen 1: 8-mo regimen; Regimen 2: 6-mo regimen. TB: Tuberculosis; HIV: Human immunodeficiency virus.

public facility due to distance from the patients home. Previous studies have used place of residence and distance from treatment centre as a proxy measures of access to care[17,30]. None of these, however, may be an appropriate indicator. Access to health services and treatment outcome is associated with a complex interplay of patient and health-provider-related factors including patient health belief model, knowledge, significant others, language, costs, and availability of local public services[5,17,19,30]. An important step, however, in improving access to care is to further expand TB and other healthcare services closer to the homes of rural patients.

Consistent with previous studies in Nigeria, Ethiopia, and elsewhere[1,31,32], SNTB and EPTB were predictors of unsuccessful outcomes. This may be because these patients have a higher frequency of HIV co-infection, and the depressed immune status results in their inability to develop an adequate immune response to control the disease[1,31]. Also, lack of cavitary lesions in their lungs makes them prone to misdiagnosis, delayed diagnosis, and higher co-morbidities often resulting in poor outcome[1,31,32]. Also, as previously documented[31,32], HIV co-infection was also a predictor of unsuccessful outcome. The reasons why HIV-infected TB patients had poorer outcomes have been speculated to be because of immunosuppression making them less able to develop adequate immune response even during treatment[31]. Furthermore, it has also been suggested that TB/HIV patients have higher rates of unsuccessful outcomes due to higher catastrophic costs of seeking care separately for HIV and TB[2,19,20], however, this needs to be confirmed in further studies. Compared to HIV-negative TB patients, higher death rates in HIV-infected TB patients was responsible for higher rates of unsuccessful outcomes among them in this study. There is therefore a need for a detailed assessment of factors responsible for death among TB/HIV patients. The WHO recommends the promotion of TB/HIV collaborative activities through: Improving mechanisms of collaboration between TB and HIV programmes, continuous surveillance of HIV among TB and vice versa, rational regimen and follow-up of TB/HIV co-infected patients[33]. Thus, scaling-up TB/HIV collaborative activities in rural settings could improve treatment outcomes.

In this study, we have shown that receiving the eight-month regimen was a predictor of unsuccessful outcomes. The new WHO guidelines recommended that this regimen be phased out[27]. Our study supports this policy in rural TB patients in a high-burden setting. Also, unlike the findings of other studies age, gender, and treatment category were not determinants of TB treatment outcomes in this study[13,15-17,31]. The reasons for these differences are not clear.

The strengths of this study are twofold: The data used were obtained under programme conditions and therefore are likely to reflect operational reality; and through several reporting and record training sessions by the TB programme there were no missing data on outcomes and we therefore believe the data were robust. However, the study had some limitations. The variables used for the analysis were derived from routine surveillance data; additional important variables such as employment status, co-morbidities like diabetes mellitus,

income levels, and adverse effects of medications could have improved our study but these information are not routinely recorded in TB registers. Also, although data on HIV status were recorded in the registers for each patient, details of $CD4^+$ T cell count, antiretroviral therapy use and cotrimoxazole preventive therapy use were not adequately documented in the registers. These could have affected treatment outcomes. A prospective study with these additional details documented will improve upon these limitations.

In conclusion, treatment success rate among TB patients in rural Nigeria was 75.7%, and receiving care at an urban public facility, smear-negative or extrapulmonary TB, HIV co-infection, and receiving the eight-month regimen were predictors for unsuccessful outcomes. These findings have implications that could modify the National TB Control Programme policy. We recommend that: (1) urgent measures should be adopted to reduce default and deaths among TB patients especially TB/HIV patients; (2) there is need to further expand quality TB education, services and TB/HIV collaborative activities in rural Nigeria; and (3) targeted interventions to reduce unsuccessful outcomes for patients in the high-risk groups should be implemented.

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COMMENTS

Background

Rural residence is marker of poverty. Thus, addressing poverty in tuberculosis (TB) control should include the needs of vulnerable, disadvantaged, marginalized sections of the population like TB patients residing in rural areas. The authors investigated the treatment outcomes of TB and the determinants of adverse outcomes in rural Nigeria.

Research frontiers

Only few studies have investigated the outcomes and its determinants of TB treatment in rural high burden settings. Knowledge of this information is needed for developing health systems and policy solutions for persons with TB residing in rural areas in resource-limited settings.

Innovations and breakthroughs

In this study, the authors found that treatment success rate among rural TB patients was very poor - which was mainly due to death or default during treatment. These poor outcomes were highest among persons who received care at the urban facility, those who had smear-negative TB, extrapulmonary TB, human immunodeficiency virus (HIV) co-infection, and patients who received the (8-mo) anti-TB regimen.

Applications

This observational study suggests that there is need to improve treatment success rate of TB in Nigeria. This may require targeting individuals who came for care at the urban facility, those who were HIV co-infected, and patients who received the longer (8-mo) anti-TB regimen for intervention.

Peer-review

This manuscript investigated outcomes of TB treatment and analyzed factors for unsuccessful TB outcomes in rural Nigeria. The manuscript is basically well written. Although this study has some limitations as authors stated in Discussion section, the findings are useful to improve TB treatment outcomes in area similar to rural Nigeria.

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ORIGINAL ARTICLE

Observational Study

Efficient management and maintenance of ultrasonic nebulizers to prevent microbial contamination

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Abstract

AIM: To seek the cause of *Burkholderia cepacia* complex (Bcc) infection outbreak and evaluate the efficacy of new methods for nebulizer maintenance.

METHODS: We investigated the annual number of Bcc isolates recovered from clinical samples in our hospital between 1999 and 2013. Swab samples were randomly collected for bacterial culture before patient use from 10 each of the two machine types in August 2001; these included 20 samples from each of the following: Drain tubes, operating water chambers, oscillators, and nebulizing chambers. In addition, 10 samples each of nebulizer solutions before and after use were cultured. For environmental investigation, 10 samples were collected from sinks in the nurse stations of the wards where patients positive for Bcc were hospitalized. Numbers of Bcc isolates were compared before and after introduction of new methods for nebulizer maintenance in October 2001. In addition, randomly amplified polymorphic DNA (RAPD) assay was applied to find the genetic divergence of the Bcc isolates obtained from clinical samples and nebulizers.

RESULTS: From January 1999 to December 2013, a

total of 487 Bcc isolates were obtained from clinical specimens from 181 patients. Notably, 322 (66.1%) Bcc isolates were obtained from clinical specimens from 1999 to 2001, including 244 (115 patients) from sputum and 34 (11 patients) from blood. During this period, 14 isolates were obtained from nebulizer components. Among these, six were derived from nebulizer drain tubes, five from operating water chambers, and one from the oscillator before patient use, and two from nebulizer solutions after patient use. When Bcc was isolated from the nebulizer solution after patient use, Bcc was simultaneously detected in other parts of the nebulizer. Bcc was not isolated from any nebulizer solution before use. RAPD assays revealed similar DNA profiles in isolates obtained from patients and nebulizers. Investigation revealed damaged diaphragms in many nebulizers. The new maintenance methods for nebulizers, including restriction of the usage period, thorough disinfection, and routine check for diaphragm breakage, remarkably reduced Bcc isolation (165 isolates from patients in 12 years and 0 isolate from nebulizers in periodical sampling). In particular, Bcc has been isolated from blood from only one patient since the new methods were introduced.

CONCLUSION: Appropriate maintenance of ultrasonic nebulizers is crucial for preventing Bcc contamination of nebulizers and subsequent respiratory tract and blood infections.

Key words: Prevention; Contamination; *Burkholderia cepacia*; Randomly amplified polymorphic DNA assay; Ultrasonic nebulizer

Core tip: In this study, we sought the cause of an outbreak of *Burkholderia cepacia* complex (Bcc) infection among inpatients using ultrasonic nebulizers and evaluated the efficacy of new methods for nebulizer maintenance introduced following the outbreak. Precise investigation revealed damaged diaphragms in many nebulizers, which we speculated would be the major cause of Bcc contamination of nebulizers and subsequent Bcc infection. The new maintenance methods for nebulizers, including restriction of the usage period, thorough disinfection, and routine check for diaphragm breakage, remarkably reduced Bcc isolation from nebulizers and patients' samples.

Ida Y, Ohnishi H, Araki K, Saito R, Kawai S, Watanabe T. Efficient management and maintenance of ultrasonic nebulizers to prevent microbial contamination. *World J Methodol* 2016; 6(1): 126-132 Available from: URL: http://www.wjgnet.com/2222-0682/full/v6/i1/126.htm DOI: http://dx.doi.org/10.5662/wjm.v6.i1.126

INTRODUCTION

Nebulizer devices are widely used to deliver aerosol therapy, especially in patients with respiratory disease[1,2]. However, nebulizers are potential sources of microbial contamination of the respiratory tract[3,4]. Small-volume medication nebulizers for administering bronchodilators, including hand-held nebulizers, can produce bacterial aerosols[5]. Both jet and ultrasonic hand-held nebulizers have been associated with nosocomial pneumonia[6-9].

The Centers for Disease Control and Prevention (CDC) established guidelines for preventing nosocomial pneumonia in 1997[10]. The guidelines specified that small-volume medication nebulizers should be disinfected, rinsed with sterile water, or air-dried between treatments on the same patient. These guidelines were substantially revised in 2003 to make these procedures (cleaning, disinfecting, rinsing, and air-drying) mandatory for maintaining medication nebulizers between treatments on the same patient[11]. However, there have been no subsequent guidelines offering more detail regarding cleaning and disinfection of nebulizers.

From 1995 to 1996, our tertiary care university hospital experienced two *Burkholderia cepacia* complex (Bcc) outbreaks associated with microbial contamination of ultrasonic nebulizer solutions[12]. Because the nebulizer solution was identified as the source of contamination, we controlled the outbreaks by replacing the nebulizer solution after each use. However, we again experienced increased numbers of Bcc isolates from sputum and blood culture from 1999 to 2001. This Bcc re-emergence forced us to re-examine the source of microbial contamination in the infected patients and to develop new methods to control the infection.

For this purpose, we compared the number of Bcc isolates from patients and nebulizers before and after introduction of a new disinfection method and analysed the genetic association between these isolates. In addition, we evaluated the efficacy of these new methods to prevent microbial contamination of ultrasonic nebulizers.

MATERIALS AND METHODS

Overview

Kyorin University Hospital is a tertiary care hospital in Tokyo, Japan, with 1153 beds. From January 1999 to December 2013, we investigated the annual number of Bcc isolates recovered from clinical samples (sputum, blood, catheter, pus, and urine) sent to our clinical laboratory in routine clinical practice. After detecting a yearly increase in Bcc isolates from 1999 to 2001, we used microbial and genetic analyses to examine routes of contamination. In many cases, nebulizers were suspected as probable sources of contamination; we therefore thoroughly investigated nebulizers to discover the main cause of microbial contamination. Furthermore, we developed new methods for maintaining nebulizers and compared the number of Bcc isolates detected before and after introducing the new methods.

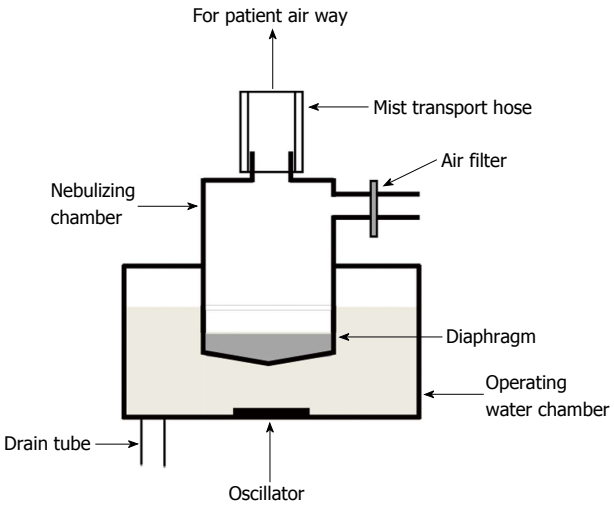

Figure 1 Components and structure of an ultrasonic nebulizer.

Ultrasonic nebulizer

In our hospital, two types of ultrasonic nebulizers, the SONICLIZER 305 (ATOM Co., Tokyo, Japan) and ULTRASONIC NEBULIZER UN-701 (Alfresa Co., Tokyo, Japan), are used for respiratory care. Both nebulizers consist of a mouthpiece, mist transport hoses, nebulizing chamber, diaphragm, operating water chamber, oscillators, drain tubes, and air filter (Figure 1). The nebulizer solution and mouthpiece are single-use, but the other parts are reused. We checked patient medical records to determine whether an ultrasonic nebulizer was used for patients from whom Bcc was isolated. We also investigated the frequency of ultrasonic nebulizer usage and maintenance from 1999 to 2005.

Operational management and maintenance for ultrasonic nebulizers

Before August 2001, nurses or helper staff disinfected ultrasonic nebulizer components, including the diaphragm, mist transport hoses, and mouthpiece, according to the manufacturer's operation instructions. The nebulizer solution was replaced with each use. All ultrasonic nebulizers were maintained in each ward and returned to the medical engineering section only when damaged.

With some modification, the CDC guidelines for preventing nosocomial pneumonia were applied from September 2001[11]. In addition to nurses or helper staff cleaning, disinfecting, rinsing with sterile water, and air-drying nebulizer components, new contamination control measures were implemented as follows: (1) Availability of ultrasonic nebulizers was tightly limited to < 5 d. At that time, the devices were returned to the medical engineering section for maintenance; (2) Nebulizer drain tubes and oscillators were completely disinfected once every 24 h using 85% ethanol; and (3) After each use, nebulizers were surveyed for diaphragm breakage or pinholes using a device that measured electrical resistance (Figure 2).

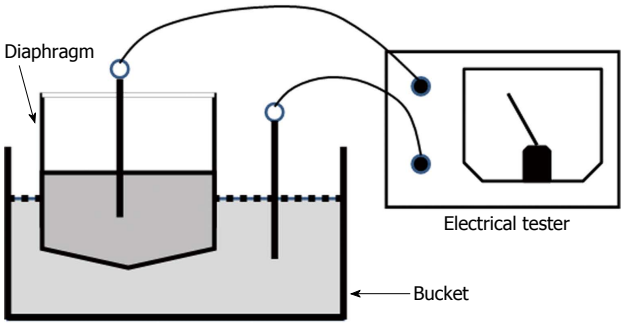

Figure 2 Scheme for discovering diaphragm damage using an electrical tester. A low concentration of detergent is added to the water in the diaphragm and bucket. Diaphragm breakage or pinholes are detected by measuring the electricity between the diaphragm and bucket.

Sampling from ultrasonic nebulizers and ward environments

Swab samples were randomly collected for bacterial culture before patient use from 10 each of the 2 machine types in August 2001; these included 20 samples from each of the following: Drain tubes, operating water chambers, oscillators, and nebulizing chambers. In addition, 10 samples each of nebulizer solutions before and after use were cultured. After the new ultrasonic nebulizer disinfection measures were implemented in September 2001, we performed a bacterial culture survey of drain tubes and oscillators of 10 nebulizer machines before patient use three times between January 2002 and December 2004. For environmental investigation, 10 samples were collected from sinks in the nurse stations of the wards where patients positive for Bcc were hospitalized. Sample solutions were centrifuged for 10 min at 3000 rpm and the resultant pellets processed for culture.

Identification and molecular typing of Bcc isolates

All clinical samples and pellets were inoculated onto 5% sheep blood agar (Oriental Yeast Co., Tokyo Japan) and incubated for 48 h at 35 ℃ in a humidified atmosphere. Bcc isolates were identified by an analytical profile index procedure using the API 20NE system (API-BioMerieux, La Balme les Grottes, France).

The genetic profiles of Bcc isolates obtained from clinical samples and nebulizers were compared using the random amplified polymorphic DNA (RAPD) assay as described previously[12]. Briefly, total DNA was prepared by boiling, and 50 ng of DNA was subjected to random polymerase chain reaction (PCR) using two PCR primers, RPKHM1 and RPKHM2, synthesized in-house. PCR products were electrophoresed in a 3% agarose gel, and the bands visualized by ultraviolet light.

RESULTS

Number of Bcc isolates

From January 1999 to December 2013, a total of 487 Bcc isolates were obtained from clinical specimens from

Table 1 Distribution of *Burkholderia cepacia* complex isolates according to specimen sources from inpatients between 1999 and 2013

	No. of isolates in each year															
	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Total
Sputum	55	66	123	80	18	12	6	10	0	4	5	1	6	3	3	392
Blood	7		27		3											37
Catheter		3	23	2	1											29
Pus	3	1	10		4		2				2	2			1	25
Urine		4														4
Total	65	74	183	82	26	12	8	10	0	4	7	3	6	3	4	487

181 patients (Table 1). Retrospective review of medical records revealed that > 90% had used a nebulizer. Notably, 322 (66.1%) Bcc isolates were obtained between January 1999 and December 2001. These included 244 isolates from sputum specimens (115 patients) and 34 from blood specimens (11 patients). After introduction of the new methods of operational management and ultrasonic nebulizer maintenance in 2001, the number of Bcc isolates from clinical specimens decreased dramatically (165 isolates in 12 years). In particular, Bcc has been isolated from blood from only one patient since the new methods were introduced. During the entire study period, the annual number of inpatients did not change remarkably, with a minimum of 291551 and a maximum of 309127 patients.

Bcc isolated from ultrasonic nebulizers and environmental samples

In the August 2001 investigation, 14 Bcc isolates were obtained from 5 nebulizers. Among these, 6 were derived from nebulizer drain tubes, 5 from operating water chambers, 1 from the oscillator before patient use, and 2 from nebulizer solutions after patient use. When Bcc was isolated from the nebulizer solution after patient use, Bcc was simultaneously detected in other parts of the nebulizer. Bcc was not isolated from any nebulizer solution before use. In contrast, after introducing the new ultrasonic nebulizer maintenance methods, Bcc was not isolated from any ultrasonic nebulizer components during three separate time points between January 2002 and December 2004. Bcc was also not isolated from environmental samples from the wards.

Management of ultrasonic nebulizers

Before introducing the new rules in September 2001, the frequency of ultrasonic nebulizer maintenance by medical engineers was < 900 times per year. The average duration of ultrasonic nebulizer usage in each ward was 34.9 d. After introducing the new rules in September 2001, the maintenance frequency increased to > 3500 times annually, and the average duration of usage in wards decreased to 5.1 d.

After September 2001, visual examination of individual nebulizer components during routine maintenance showed obvious breakage in 2 of 20 diaphragms. No remarkable defects were found in other nebulizer components. In addition, routine testing of diaphragms by electrical resistance revealed damage in 34 of 140 nebulizers. After introduction of the new rules, these damaged diaphragms were not used on patients.

Genotypic analysis of Bcc isolates

RAPD analysis revealed 15 fingerprint patterns, designated R6 to R20, among 140 isolates randomly chosen from patient specimens collected between January 2000 and July 2003. The genetic profiles of endemic strains from the most recent outbreak differed from the previous outbreak (RAPD profiles R1 to R5) from November 1995 to September 1996 (data not shown)[12]. Among DNA profiles R6 to R20, R6 was most prevalent, detected in 102 isolates (71.4%). The next most prevalent pattern, R7, was detected in 15 isolates (10.7%). Patterns R8 to R10 were detected in 3 isolates each, R11 to R14 in 2 each, and R15 to R20 in 1 each.

Among 14 isolates from nebulizers, 6 fingerprint patterns, R6, R7, R8, R21, R22 and R23, were observed. Four isolates had an R6 pattern; 1 was R7; 6 were R8; and 1 each were R21, R22, and R23 (Figure 3). DNA fingerprint patterns of each component matched those from nebulizer solutions when Bcc was isolated from both samples. Since 2003, we have not performed genotypic analyses of Bcc isolates because isolates were only sporadically recovered from patients in different wards.

DISCUSSION

This investigation, together with our previous reports regarding a Bcc outbreak, clearly showed that Bcc was harboured in ultrasonic nebulizers and caused respiratory tract infections in patients using them. Bcc, a ubiquitous bacterial species in the natural environment, is capable of surviving and growing in nutrient-poor water[13]. Bcc has been recovered from hospital environments, including sink drains and hospital tap water, medical devices including nebulizers, and a variety of solutions used in clinical practice[14-17]. Therefore, disinfection of ultrasonic nebulizer components after use is important for preventing Bcc contamination. To date, public guidelines contain no precise details regarding methods for disinfecting nebulizers. According to the manufacturers' instructions, the components, including oscillators, should be wiped and disinfected with a

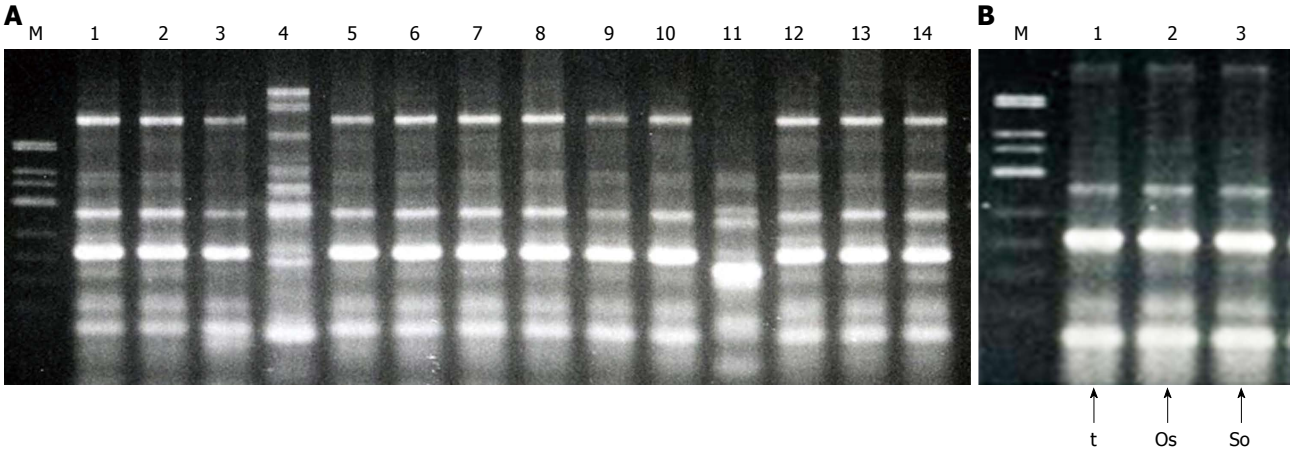

Figure 3 DNA fingerprints of strains determined by random amplified polymorphic DNA assay. A: Isolates from each patient (1-14); B: Isolates from nebulizer components. t: Nebulizer drain tube; Os: Oscillator; So: Nebulizer solution; M: DNA size marker.

0.1%-0.5% benzalkonium chloride aqueous solution. A previous study demonstrated that disinfection at 24-h interval is indispensable when nebulization solutions not containing preservatives are used[4]. However, because of their complicated structures, nebulizer components are difficult to keep dry, and wiping with disinfectant detergent might lead to incomplete disinfection. In addition, nebulizers may be used on additional patients before the oscillator is completely dried and disinfected, as is the situation in our tertiary care hospital where frequent nebulizer use is required. Furthermore, Bcc can form biofilms[18]. Biofilms on nebulizer components may interfere with effective disinfection of Bcc.

In the present study, prior to introducing new measures for operational management and maintenance of nebulizers, Bcc was isolated from drain tubes and oscillators before use and from the nebulizer solution after use, but not from the nebulizer solution before use. These data suggest that Bcc initially contaminated nebulizer components, with secondary contamination of the nebulizer solution. Notably, diaphragm breakage or pinholes were found in multiple nebulizers using a precise investigation. Diaphragm breakage can allow microorganisms to invade the nebulizing chamber and contaminate the nebulizer solution. Thus, we speculated that small diaphragm breakage or pinholes led to Bcc contamination of the nebulizer solution, causing respiratory tract infection in patients using the nebulizers. The diaphragm in an ultrasonic nebulizer is typically reused, and the thickness of its plastic bottom decreases with continuous ultrasonic wave pressure. Small diaphragm breakage and pinholes may be overlooked by visual inspection. Therefore, we introduced new methods using electrical devices to check for diaphragm breakage. In addition, the average routine medical engineer maintenance interval was shortened from 34.9 to 5.1 d. Since introducing these new methods, the number of Bcc isolates from clinical specimens has decreased dramatically. These findings suggest that our new rules for maintaining nebulizers are effective in preventing nosocomial respiratory infection by Bcc.

While Bcc is considered of relatively low virulence and believed to rarely cause invasive disease, several studies have reported this microorganism to be an important infectious agent, causing bacteraemia with substantial clinical impact[19]. Jang *et al*[20] performed a prospective epidemiologic analysis of 147 nosocomial gram-negative bacteraemia episodes among intensive care units patients. The Bcc isolation rate was second only to *Acinetobacter baumannii*, and the most frequent primary infection site was the lower respiratory tract in the patients with bacteraemia. Although we could not clarify the source of Bcc bacteraemia in the present study, a substantial number of isolates were recovered from blood culture along with increased sputum isolates and Bcc isolation from multiple nebulizer components between 1999 and 2001. After introducing new methods for maintaining nebulizers, no Bcc bacteraemia was detected, in concordance with the absence of Bcc in nebulizer samples. These results suggest that disinfecting nebulizers is crucial for preventing Bcc bacteraemia and subsequent respiratory tract infections in patients using nebulizers.

Previous studies have verified that RAPD is a powerful tool for identifying routes of microbial infection, including Bcc, in nosocomial infections[21,22]. In the present study, genotypes of Bcc isolated from nebulizer components were similar to isolates from patients using nebulizers, but distinct from those from the previously recognized Bcc outbreak. These data suggest that contamination of nebulizer components is responsible for respiratory and bloodstream infections by Bcc in these patients. This observation confirms our previous report that the RAPD assay is useful for identifying the source of nosocomial Bcc infection.

Our investigation confirmed that nebulizers are important sources of Bcc contamination, which causes respiratory tract infection and subsequent bacteraemia. Our findings suggest that appropriate operational management and ultrasonic nebulizer maintenance are crucial for preventing microbial contamination of nebu-

lizers and subsequent respiratory tract and bloodstream infections. Furthermore, RAPD is a powerful tool for identifying routes of nosocomial Bcc infection.

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COMMENTS

Background

Nosocomial infection is a ubiquitous problem in healthcare facilities. An ultrasonic nebulizer is one of the potential sources of microbial contamination of the respiratory tract and subsequent infection of lung and blood stream among the patients using this equipment. However, public guidelines containing precise details regarding methods for disinfecting nebulizers have yet been established to date. Therefore, proper methods for management of usage and disinfection of nebulizers need to be developed.

Research frontiers

The Centers for Diseases Control and Prevention established guidelines for preventing nosocomial pneumonia in 1997 and revised them in 2003. In the guidelines, cleaning, disinfecting, rinsing, and air-drying of the nebulizers are prescribed to be mandatory between treatments on the same patient. However, the clinical relevance of these procedures has yet been tested in clinical settings.

Innovations and breakthroughs

A few studies have addressed the issues regarding bacterial contamination of nebulizers, and no detailed procedure except for the frequent disinfection has been emphasized in the previous studies. The present study specified breakage of diaphragm as an important cause for bacterial contamination of nebulizers. Furthermore, the efficient method for detecting breakage of diaphragm using an electrical device has been developed in this study.

Applications

This study allows readers to perform appropriate maintenance and disinfection of ultrasonic nebulizers, and will contribute to the decrease of nosocomial infection of respiratory tract and blood stream, at least that by *Burkholderia cepacia* complex (Bcc) which is described as a main pathogen transmitted by nebulizers in this study.

Terminology

Burkholderia cepacia is a gram-negative rod previously known as *Pseudomonas cepacia*. While Bcc is considered of relatively low virulence and believed to rarely cause invasive disease, several studies have reported this microorganism to be an important infectious agent, causing bacteraemia with substantial clinical impact.

Peer-review

This is interesting and well written article, which may be a useful source of knowledge for all clinicians, because nosocomial infections are an important problem of contemporary clinical practice. The research is well designed and experimental part is described in detail.

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